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Review

Mechanism of fatty acid synthesis*

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[Received for publication August 3, 1960]

For many years one of the predominating concepts in biochemistry has been the general assumption that synthetic and degradative processes are alternate aspects of a reversible mechanism. The thesis was widely accepted and was thought to include processes involved in the synthesis of proteins, carbohydrates, fats, etc. However, this hypothesis has lost most of its attractiveness; in every instance in which it was thought to apply, experiments proved the contrary.

Fatty acid synthesis is an excellent example. Until a few years ago (1 to 12), the predominant concept of the mechanism of fatty acid synthesis was that it occurred via the reversal of the enzymatic reactions involved in β-oxidation. This concept was advocated by Lynen early in 1953 (13), when he stated, "The β-oxidation of fatty acids proposed by Knoop is nothing else but the reversal of this (synthetic) cyclic process."

This concept of fatty acid synthesis was generally accepted by the biochemical community despite earlier observations by Gurin and his colleagues (14, 15, 16) on the possible presence of two separate and distinct systems for synthetic and degradative processes. Experimental evidence supporting fatty acid synthesis via "a modified scheme for reversal of β-oxidation sequence" came from two independent observations. The first was the discovery by Langdon (17) that crotonyl CoA can be reduced by TPNH in the presence of an enzyme found in the soluble extracts of rat liver; the second was the demonstration by Stumpf and Barber (18) and by Wakil *et al.* (19) that stearic acid synthesis from acetyl CoA and palmityl CoA was catalyzed by mitochondrial enzymes in the presence of DPNH and TPNH. Pyridoxal phosphate (19) appears to be a cofactor in this conversion, a fact which is supported by nutritional studies relating to the *in vivo* synthesis of long-chain fatty acids.

The afore-mentioned system is located in the mitochondria and is concerned primarily with the elongation of fatty acids of moderate chain length. Thus the name "mitochondrial," or "elongation," system for fatty acid synthesis has been proposed for this series of reactions.

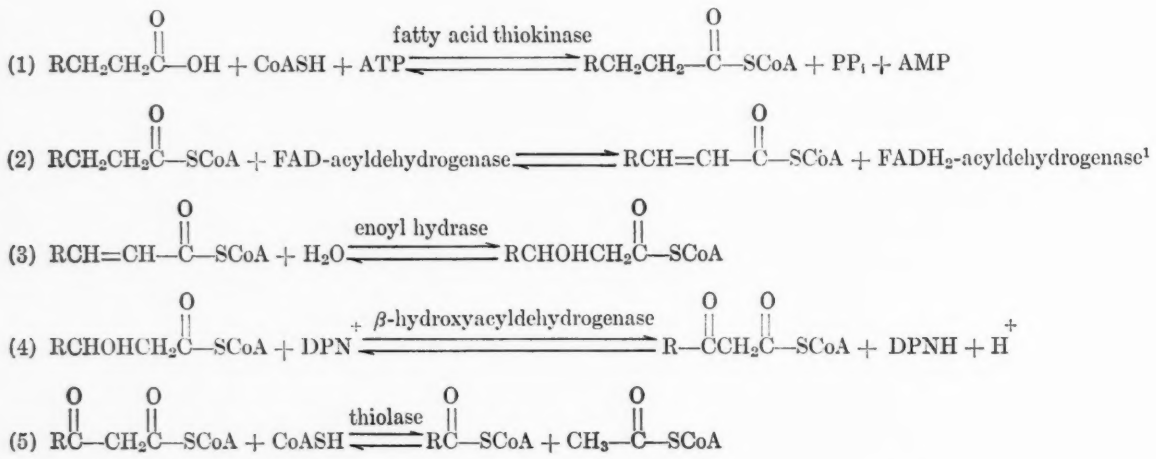
Evidence for a second pathway of fatty acid synthesis was obtained at the Enzyme Institute at the University of Wisconsin (5, 6, 7, 9, 10) by the isolation of a new enzyme system from avian liver that was free of the key enzymes of the β-oxidation sequence. This system converted acetyl CoA to long-chain fatty acids in the presence of ATP, Mn^{++}, CO_2, and TPNH. It appeared to be associated with particles of a size smaller than microsomes (the whole system may be separated as a pellet on centrifugation at $140{,}000 \times g$ for 2 hours) since neither mitochondria nor microsomes contain this system. Furthermore, malonyl CoA is the key intermediate (9, 10) in this system and is the compound which contributes all but one of the C_2 units going into the final acyl CoA product. For these reasons, the name "non-mitochondrial system" or " malonyl CoA pathway" for fatty acid synthesis has been proposed.

This review deals with some new aspects of these two systems and summarizes our present views as to the mechanism of synthesis of fatty acids.

* Aided in part by Grants RG-6242 (C1), H-3582, and M-2109 from the National Institutes of Health, United States Public Health Service, and grants from the Center for the Study of Aging, Duke University, the American Cancer Society, and the Life Insurance Medical Research Fund.

THE "MITOCHONDRIAL SYSTEM" FOR FATTY ACID SYNTHESIS

In 1953, several laboratories (20 to 23) announced the elucidation of the mechanism of fatty acid oxidation, demonstrating that fatty acids are oxidized via a pathway very similar to the β-oxidation scheme proposed by Knoop (24) fifty years earlier. The sequence of the reactions and the enzymes involved are as follows:


(1) RCH2CH2C(=O)—OH + CoASH + ATP ⇌ (fatty acid thiokinase) RCH2CH2—C(=O)—SCoA + PPi + AMP
(2) RCH2CH2C(=O)—SCoA + FAD-acyldehydrogenase ⇌ RCH=CH—C(=O)—SCoA + FADH2-acyldehydrogenase[1]
(3) RCH=CH—C(=O)—SCoA + H2O ⇌ (enoyl hydrase) RCHOHCH2C(=O)—SCoA
(4) RCHOHCH2C(=O)—SCoA + DPN+ ⇌ (β-hydroxyacyldehydrogenase) R—C(=O)CH2C(=O)—SCoA + DPNH + H+
(5) RC(=O)—CH2C(=O)—SCoA + CoASH ⇌ (thiolase) RC(=O)—SCoA + CH3—C(=O)—SCoA

Each of the enzymes involved in this sequence of reactions has been prepared in a highly purified state and each step has been shown to be reversible. The equilibrium constants for the first four reactions were shown to be near unity, whereas the thiolase reaction has an equilibrium constant of 1.6×10^{-5} at pH 7.0 (25); this means that in the presence of CoASH there is very little β-ketoacyl CoA present. Nevertheless, the equilibrium can be shifted toward condensation in the presence of DPNH and β-hydroxyacyl dehydrogenase.

Stadtman and Barker (26 to 32) were the first to demonstrate the conversion of labeled acetate into short-chain fatty acids by water-soluble enzyme preparations which they obtained from *Clostridium Kluyveri*. Short-chain fatty acids (butyrate and hexanoate) were synthesized by the *Kluyveri* extract and the reactions from acetyl CoA to butyryl CoA and hexanoyl CoA were catalyzed presumably by the enzymes of the β-oxidation sequence.

[1] The reduced acyldehydrogenase is then oxidized by the electron transferring flavoprotein to an FAD-acyldehydrogenase and reduced electron transferring flavoprotein (77). The latter enzyme then transfers its electron to a suitable acceptor (cyctochrome C or a dye).

Later, Stansly and Beinert (33) demonstrated the conversion of acetyl CoA to butyryl CoA in the presence of DPNH, a reduced dye, and the purified enzymes of the fatty acid oxidation cycle. They were unable to demonstrate the formation of significant amounts of longer acyl CoA derivatives. This appeared to be the result of a preferential condensation of acetyl CoA with another molecule of acetyl CoA rather than with a higher homologue (butyryl CoA, hexanoyl CoA, etc.). An additional weakness in these experiments was the requirement of reduced dye as an electron donor rather than a natural electron donor such as reduced pyridine nucleotides, reduced flavins, reduced cytochromes, etc.

In 1955, Langdon (17, 34) discovered, in soluble extracts of rat liver, an enzyme (TPNH crotonyl CoA reductase) that catalyzed the reduction of crotonyl CoA by TPNH. Later, Seubert *et al.* (35) were able to isolate this enzyme from pig liver mitochondria and to purify it extensively by differential centrifugation in the presence of cholate. The purified particulate enzyme was free from the various enzymes of the β-oxidation cycle and had a wide range of specificity, ranging from crotonyl CoA to α,β-unsaturated stearyl CoA. The localization of this enzyme in the mitochondria is of extreme interest to our knowledge of the cellular distribution of the fatty acid synthesizing systems. With the aid of this enzyme, Seubert *et al.* (35) were able to reconstitute a fatty acid synthesizing system from the purified enzymes of the β-oxida-

tion cycle (thiolase, enoyl hydrase, and β-hydroxyacyl dehydrogenase), a source of DPNH (alcohol and alcohol dehydrogenase), and a source of TPNH (glucose-6-phosphate and glucose-6-phosphate dehydrogenase). With this system they were able to demonstrate the synthesis of octanoyl and capryl CoA from hexanoyl CoA and acetyl-1-C^{14} CoA. However, the poor yield of naturally occurring longer chain fatty acids, e.g., stearic and palmitic, suggested that this pathway for fatty acid synthesis may not be the operational one *in vivo*.

Stumpf and Barber (18) were the first to show the fatty acid synthesis in intact mitochondria derived from avocado mesocarp. Adenosine triphosphate, CoA, and Mn^{++} were essential components for the conversion of C^{14}-acetate into the long-chain fatty acids (palmitic and oleic) in this system.

Recently, Wakil *et al.* (19) were able to demonstrate that intact mitochondria isolated from pigeon, rat, or beef livers can synthesize long-chain fatty acids from acetyl CoA (Table 1). Mitochondria were prepared in either 0.25 M or 0.88 M sucrose according to the general procedures of Hogeboom *et al.* (36). When the mitochondria were incubated anaerobically with acetyl-1-C^{14} CoA, ATP, DPNH, and TPNH, C^{14}-labeled long-chain fatty acids were isolated. Under these conditions, the conversion of acetyl CoA to fatty acids was completely dependent upon and directly related to the presence of ATP (Fig. 1). In the presence of 10 μmoles of ATP, 10% to 20% of the acetyl CoA was converted to fatty acids. Neither butyryl CoA nor GTP could substitute for ATP, whereas ADP, CTP, and UTP could replace ATP at relatively high levels.

TABLE 1. FATTY ACID SYNTHESIS BY MITOCHONDRIA

	Acetyl CoA Incorporated into Long-Chain Fatty Acids
	μmoles
Complete system*	4.5
No ATP	0.3
No TPNH	2.4
No DPNH	2.0
No TPNH; no DPNH	0.0
Complete system + HCO_3^-	3.0
No acetyl CoA + malonyl CoA	2.4
No acetyl CoA + malonyl CoA, without ATP	0.8

* The reaction mixture contained 34.5 mμmoles acetyl CoA (55,200 cpm), 8 μmoles ATP, 0.25 μmole TPNH, 0.25 μmole DPNH, 2.8 mg of mitochondrial protein, 50 μmoles phosphate (pH 6.5), and H_2O to a total volume of 0.5 ml. Four μmoles $KHCO_3$ and 30 mμmoles malonyl CoA (HOOC $CH_2C^{14}O$ CoA 70,000 cpm) were used where indicated. The reaction mixture was incubated anaerobically at 38° for 1 hour.

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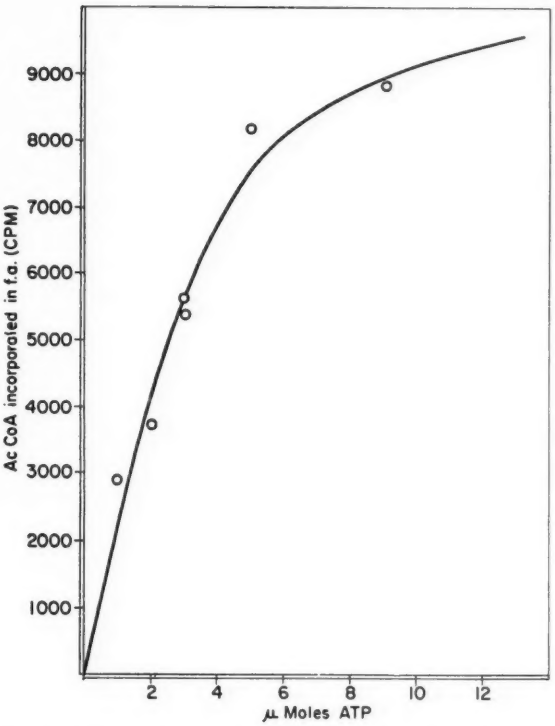


FIG. 1. ATP requirement in fatty acid synthesis by mitochrondria. The reaction mixtures contained 34.5 mμmoles of acetyl CoA (55,200 cpm), 0.25 μmole TPNH, 0.25 μmole DPNH, 2.8 mg of mitochrondrial protein, 50 μmoles phosphate (pH 6.5), ATP as indicated, and H_2O to a final volume of 0.5 ml. The reaction mixture was incubated anaerobically at 38° for 1 hour.

The incorporation of acetyl CoA into the fatty acids was proportional to the amounts of mitochondria added, as is shown in Figure 2. Anaerobic conditions were necessary to minimize the oxidation of DPNH and TPNH by molecular oxygen. Both reduced pyridine nucleotides (TPNH and DPNH) were required for optimum synthesis; in the absence of either reduced nucleotide there was 60% to 80% inhibition of synthesis, while in the absence of both nucleotides there was no synthesis at all.

The omission of HCO_3^- from this system did not affect the synthesis, in contrast to the non-mitochondrial system which is absolutely HCO_3^- dependent

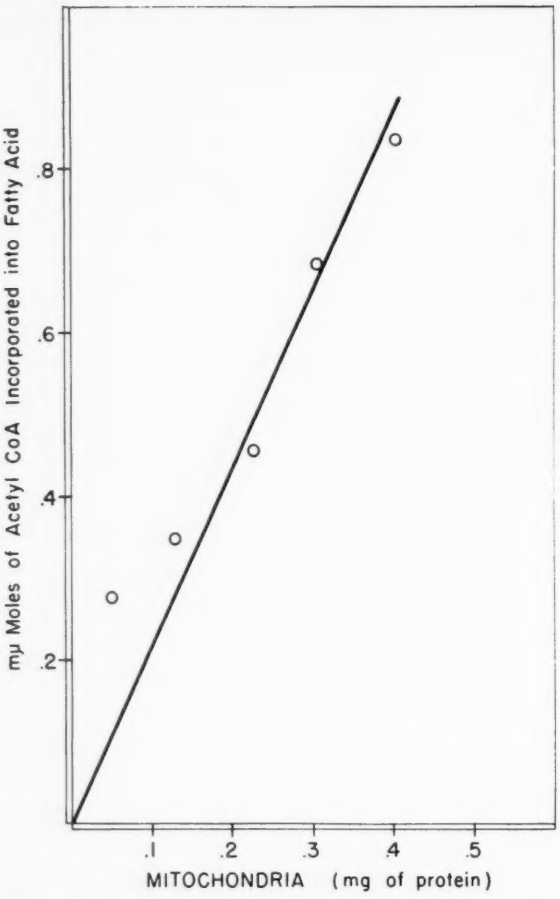


FIG. 2. The relationship between C^{14}-acetyl CoA incorporation in fatty acids and the amount of mitochondria. Each reaction contained 58.6 mμmoles 1-C^{14}-acetyl CoA (152,000 cpm), 0.1 μmole TPNH, 0.1 μmole DPNH, 2 μmoles ATP, 30 μmoles phosphate buffer (pH 6.5), and water to a final volume of 0.5 ml. The reaction was started with the addition of freshly prepared rat liver mitochondria as indicated. The reaction mixture was incubated anaerobically at 38° for 1 hour.

(Table 5). Furthermore, C^{14}-malonyl CoA was incorporated into the fatty acids only in the presence of ATP (Table 1). Malonyl CoA is known to be decarboxylated to acetyl CoA by a mitochondrial enzyme (37), and it seems possible that this accounts for the utilization of malonyl CoA in the presence of ATP.

The products from the incorporation of acetyl-1-C^{14} into fatty acids by mitochondria were separated from the reaction mixture by the procedure of Wakil *et al.* (1) and were identified by a variety of techniques (38, 39). Figure 3 shows the distribution of the radioactivity among the various acids (C_{12} to C_{20}) when separated by reverse-phase chromatography according to the procedure of Kaufmann and Nitsch (39). The major components of the synthesized fatty acids were stearic acid (40%) with C_{20} acid, palmitate, myristate, and laurate constituting the remainder. This is in sharp contrast to the non-mitochondrial system where palmitic acid is the only end product of the reaction.

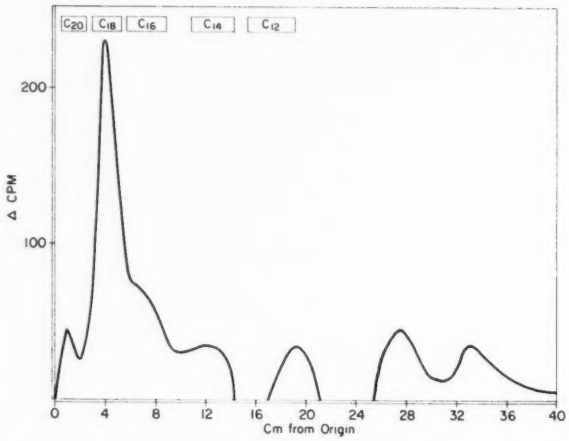


FIG. 3. Distribution of the mitochondria-synthesized fatty acids in the paper chromatographic system of Kaufmann and Nitsch (39).

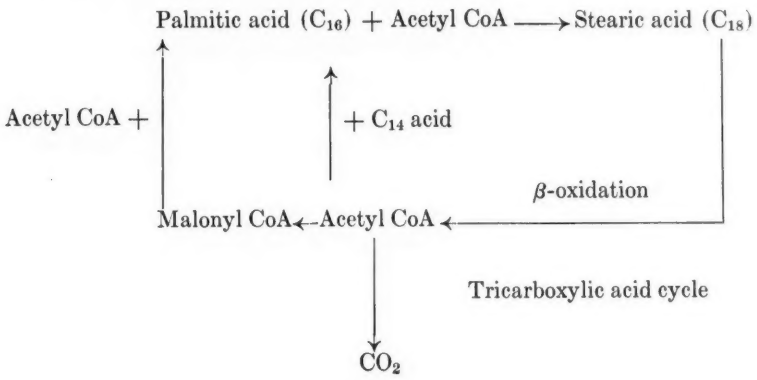
Stearic acid synthesized from 1-C^{14}-acetyl CoA was isolated by dilution with unlabeled stearic acid, recrystallized from various solvents to a constant specific activity, and the resulting acid decarboxylated by the Schmidt reaction as described by Phares (40). The specific activity (cpm per microatom carbon) of the liberated CO_2 was slightly over twice the specific activity of stearic (expressed as cpm per microatom carbon), indicating either *de novo* synthesis of stearic acid from acetyl CoA or the elongation of a pre-existing short-chain fatty acid by the successive additions of acetyl CoA.

It is too early to ascertain which of these mechanisms is operative. Most evidence, especially that obtained from the soluble enzyme system (see below), points to an elongation of short-chain fatty acids rather than total synthesis from acetyl CoA. This again contrasts with the synthesis of palmitate from acetyl CoA by the non-mitochondrial system which is true *de novo* synthesis (3). The presence of intermediate length fatty acids (C_{16}, C_{14}, and C_{12}) in the mitochondrial system is of special significance in reference to the synthetic mechanism in the mitochondria.

These results are in complete agreement with reported experimental data on the synthesis of stearate and palmitate in whole animals (41 to 50). The significant finding by many investigators was that pal-

mitic acid (C_{16}) can be converted to stearic acid (C_{18}) by the addition of one C_2-unit, while the reverse does not appear to be possible. In other words, the conversion of stearic acid to palmitic acid is not achieved by the mere cleavage of one C_2-unit, but instead, is brought about by the complete oxidation of stearic acid to acetyl CoA and the subsequent conversion of acetyl CoA to palmitic acid via either the malonyl CoA pathway or by condensation on a pre-existing myristic acid (C_{14}). This is illustrated by the following scheme:

SCHEME 1. Relationship between palmitic and stearic acids.



The inability of stearic acid to be directly converted to palmitic acid by the loss of one "C_2-unit" may be explained by the well-known observation that a fatty acid molecule being oxidized by the β-oxidation enzymes (mitochondria or whole animal systems) is completely converted to acetyl CoA with very little, if any, chance of leaving the enzyme prior to complete oxidation (20, 50, 51, 52). Thus, once stearic acid is on the path of oxidation, it continues until it is completely converted to acetyl CoA. The resulting acetyl CoA can be either oxidized to CO_2 by the tricarboxylic acid cycle or can be channeled into the various synthetic reactions, e.g., palmitic acid, amino acids, carbohydrates, purines, etc. Palmityl CoA is synthesized primarily by the non-mitochondrial system (the malonyl pathway) and can be elongated by the mitochondria by the addition of acetyl CoA to form stearyl CoA. On this basis, therefore, the addition of C^{14}-1-acetyl CoA would result in the formation of stearic acid labeled predominantly in the carboxyl group of the molecule. This, indeed, appeared to be the case in the stearic acid synthesized by rat liver slices as reported by Zabin (49), who found that the C^{14} content of the carboxyl carbon of stearic acid derived from acetate-1-C^{14} was higher than the rest of the carbon atoms of the molecule, while the remaining C^{14} in carbon atoms 3 to 18 of stearic acid appeared to be uniformly distributed along the chain. On the other hand, palmitic acid which was isolated simultaneously from the liver slices (49) showed isotope concentration uniformly distributed along the entire molecule. These results suggest that the addition of the last two carbon atoms to palmitate (C_{16}) to form stearate (C_{18}) is accomplished by a different mechanism from the multiple condensation of the "C_2-units" to form the C_{16} molecule. The mitochondrial system appears to be concerned with such elongation of C_{16} acid and to form C_{18} acid and may account for the variation of the isotope ratio in carbons 1 and 2 of C_{18} acid as compared to the remaining carbon atoms.

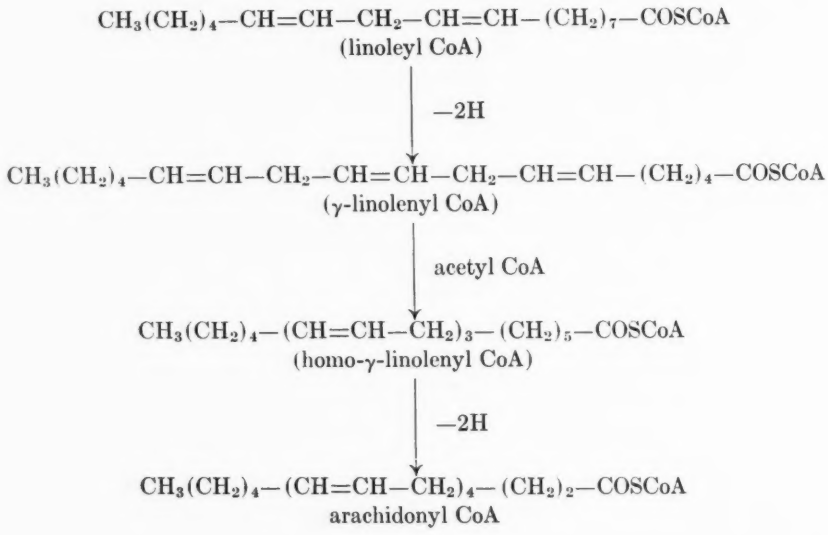
The incorporation of acetyl CoA into C_{20} fatty acids (Fig. 3) is extremely significant and may indicate that the mitochondrial system is the site for synthesis of these acids by the addition of one acetyl CoA unit onto a C_{18} fatty acid. When stearyl CoA was incubated anaerobically with 1-C^{14}-acetyl CoA, mitochondria, TPNH, and DPNH, the long-chain fatty acids were isolated and tentatively identified as a C_{20} fatty acid (possibly arachidic acid) by paper chromatography. Similar condensation of 1-C^{14}-acetyl CoA can be demonstrated with oleyl CoA as substrate instead of stearyl CoA and the formation of C_{20} acid. We have not as yet identified the C_{20} acid produced from either stearyl CoA or oleyl CoA. Neither have we any information as to whether the C_{20} produced from oleyl CoA still retains its double bond. If the C_{20} acid derived from oleyl CoA is unsaturated, and if other un-

saturated fatty acyl CoA's can substitute for oleyl CoA in this system, then the *in vivo* experiments of Mead and his colleagues (53 to 57) on the synthesis of arachidonic acid from C^{14}-labeled stearic, linoleic, and linolenic acids would be in accordance with this hypothesis. According to Mead, linoleic acid is dehydrogenated to γ-linolenic acid, which then adds acetyl CoA to homolinolenic acid; the latter acid is subsequently dehydrogenated to arachidonic acid. The over-all scheme proposed by Mead and Howton is as follows: (CoA derivatives of these acids are probably the forms participating in these reactions):

enzymatic activity, as shown in Table 3. The enzymatic activity can be completely restored by addition of a boiled extract of the enzyme preparation. We were able to show that the boiled extract can be completely replaced by two distinct compounds: an intermediate-chain fatty acid (C_8, C_{10}, C_{12}, C_{14}, etc.) and pyridoxal phosphate (Table 3).

The requirement for the addition of fatty acid acceptor indicated that this system causes the elongation of fatty acids by the addition of an acetyl CoA unit to an appropriate fatty acid. For example, when C_8 acid was used as an acceptor, we were able to isolate

SCHEME 2. Possible pathway for the conversion of linoleyl CoA to arachidonyl CoA.



Elongation of Fatty Acids by Soluble Extracts of Mitochondria. When an acetone powder of liver mitochondria was extracted with buffer a particle-free extract was obtained which catalyzed the incorporation of acetyl CoA into higher chain fatty acids in the presence of ATP, DPNH, and TPNH, as shown in Table 2. In the absence of any one of these components there was little or no incorporation of C^{14}-acetyl CoA into the fatty acids, in agreement with the results obtained from intact mitochondria. The product of synthesis in the soluble system was essentially similar to that obtained by whole mitochondria, except that there was more incorporation in the relatively shorter chain acids, compared to the higher chain acids.

Dialysis of the soluble extracts and their subsequent treatment with charcoal resulted in a sharp decline in C_{10} acid; when C_{10} acid was used, a C_{12} acid was isolated, and so on. The higher fatty acids isolated in these experiments (i.e., C_{10} in the first example and C_{12} in the second) contained over 80% of the incorporated C^{14}-acetyl CoA.

When the corresponding acyl CoA derivatives (C_8 CoA, C_{10} CoA, C_{12} CoA, etc.) were used, ATP was no longer necessary (Table 3), demonstrating that the ATP was required for the formation of the acyl CoA's of these endogenous fatty acids. Butyryl CoA and hexanoyl CoA are very poor replacements for the higher chain acyl CoA's in this system, indicating a specificity for the elongation of intermediate- and long-chain fatty acids, but not for the shorter chain acids. This may be the result of either the lack of an enzyme that would catalyze the condensation of acetyl

CoA with butyryl CoA and hexanoyl CoA, or to the absence of the specific TPNH-α,β-unsaturated acyl CoA reductase for these fatty acids (19). Indeed, we could not detect the TPNH-crotonyl CoA reductase when the extract was assayed with crotonyl CoA as substrate (19). (We have not attempted, as yet, an assay for the reduction of longer chain α,β-unsaturated acyl CoA derivatives by TPNH in the soluble extract.)

The requirement for DPNH and TPNH may be explained by the need for DPNH to reduce the condensation product (β-ketoacyl CoA), while TPNH is needed for the reduction of the α,β-unsaturated acyl CoA to the saturated derivative. The over-all scheme may be written as follows, illustrating the conversion of palmityl CoA to stearyl CoA.

$$\text{(6) palmityl CoA } (C_{16}\text{CoA}) + \text{acetyl CoA} \xrightarrow{\text{I}} \beta\text{-keto-stearyl CoA} + \text{CoA}$$

$$\text{(7) } \beta\text{-keto-stearyl CoA} + \text{DPNH} + H^+ \xrightarrow{\text{II}} \beta\text{-hydroxystearyl CoA} + \text{DPN}^+$$

$$\text{(8) } \beta\text{-hydroxystearyl CoA} \xrightarrow{\text{III}} \alpha,\beta\text{-unsaturated stearyl CoA} + H_2O$$

$$\text{(9) } \alpha,\beta\text{-unsaturated stearyl CoA} + \text{TPNH} + H^+ \xrightarrow{\text{IV}} \text{TPN}^+ + \text{stearyl CoA}$$

I = condensing enzyme possibly containing pyridoxal phosphate

II = β-hydroxyacyl CoA dehydrogenase

III = enoyl hydrase

IV = α,β-unsaturated acyl CoA reductase

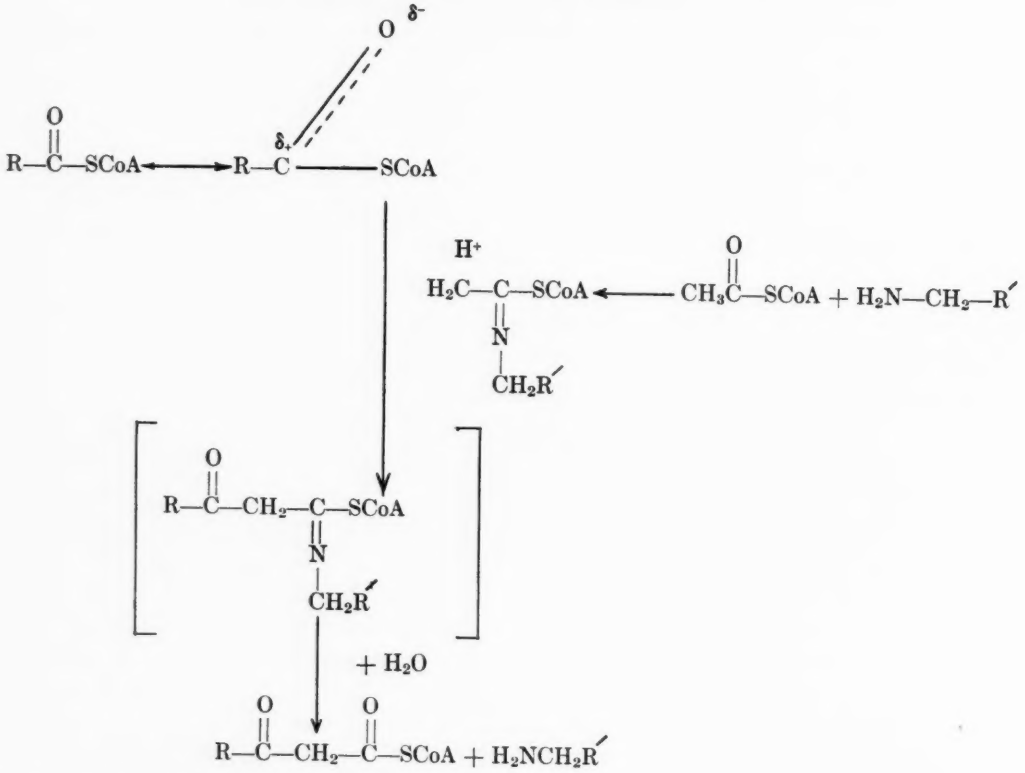
The Role of Pyridoxal Phosphate in Fatty Acid Synthesis. Birch and György in 1936 (58) found that rats suffering from vitamin B_6 deficiency developed symptoms of acrodynia similar to those obtained earlier by Burr and Burr (59, 60, 61) with fatty acid deficient rats; they also observed that unsaturated fatty acids exercised a sparing effect on vitamin B_6. These initial observations were confirmed by many workers and were extended to show that the acrodynia caused by pyridoxine deficiency can be overcome by feeding arachidonic acid or linoleic acid (62, 63, 64). Sherman *et al.* (64) were able to demonstrate an increase in the synthesis of arachidonic acid in rats receiving pyridoxine. However, Witten and Holman (63) submitted evidence to show that rats fed on a fat-deficient, pyridoxine-deficient diet developed acrodynia which can be relieved best by pyridoxine plus linoleate, suggesting that pyridoxine-deficient animals may suffer a secondary deficiency of arachidonic acid.

Preliminary observations in our laboratory indicate that the relationship between pyridoxine and essential fatty acid metabolism may be confined to the role of pyridoxal phosphate in the elongation of fatty acids. When soluble extracts of the mitochondrial system were dialyzed and treated with charcoal, a partial requirement for pyridoxal phosphate was noted (Table 3). The effect of pyridoxal phosphate can be demonstrated not only in soluble extracts but also in particles derived from mitochondria. A two- to threefold increase in the amount of C^{14}-acetyl CoA incorporated into the longer chain fatty acids was observed when the pyridoxal phosphate concentration in the reaction mixture was about 10^{-3} M. Pyridoxamine phosphate can substitute for pyridoxal phosphate but with less efficiency, whereas pyridoxine or pyridoxal hydrochloride are ineffective (Table 3). The response to added pyridoxal phosphate could be demonstrated in some, but not in all, preparations.

The precise role of pyridoxal phosphate in the proposed sequential reactions of fatty acid elongation (Scheme 2) cannot be defined at present. The most likely possibility appears to be in the condensation step of acetyl CoA with the fatty acyl CoA acceptor (65), thus avoiding the requirement of thiolase for this condensation reaction. Thiolase has two drawbacks: the first is the extremely low equilibrium constant of the condensation reaction (1.6×10^{-5} M) at pH 7.0 (25), and the second is the greater tendency of thiolase to catalyze the condensation of two acetyl CoA molecules to form acetoacetyl CoA, rather than the condensation of acetyl CoA with a higher acyl CoA

(33, 60). The involvement of pyridoxal phosphate in this reaction may overcome these drawbacks as follows: first, by the introduction of specificity to the reaction favoring the condensation of acetyl CoA with an intermediate-chain acyl CoA; and second, by the formation of a complex pyridoxal or pyridoxamine with acetyl CoA resulting in the formation of a Schiff base (Fig. 4) similar to the general pyridoxal coenzyme models proposed by Snell and his colleagues[2] (66). This may activate the methyl group of acetyl CoA by imposing an electronegative charge, thus favoring condensation with the relatively positively-charged carbonyl group of acyl CoA resulting in the elongation of the carbon chain as shown in Scheme 3:

SCHEME 3. A hypothetical mechanism of action of pyridoxal phosphate in fatty acid elongation. *R* represents the rest of the pyridoxamine phosphate molecule.



Thus the role of pyridoxal phosphate in this reaction may be as an additional means for the specific activation of the methyl group of acetyl CoA, other than the formation of malonyl CoA. This also would result in changing the equilibrium of the reaction in favor of carbon-carbon chain formation rather than cleavage.

Another alternative for the action of pyridoxal phosphate may be in the reduction of the α,β-unsaturated fatty acyl CoA derivatives by TPNH. This possibility appears very unlikely at present and must await further experimentation.

THE NON-MITOCHONDRIAL SYSTEM FOR FATTY ACID SYNTHESIS

A decade ago Gurin and his co-workers (14, 15, 16) reported the synthesis of long-chain fatty acids from acetate, first in homogenates and later in particle-free extracts prepared from pigeon liver. They found that a water extract of mitochondria was required in addition to the particle-free supernatant fluid, and, after treatment of such an extract with charcoal, could demonstrate a requirement for ATP, DPN^+, and CoA. The addition of citrate to this system stimulated the process, as did Mg^{++}; acetyl CoA, however, was less

[2] For comprehensive reviews on this subject, see Snell (66), Westheimer (103), and Braunstein (104).

TABLE 2. FATTY ACID SYNTHESIS BY SOLUBLE MITOCHONDRIAL EXTRACTS

	Acetyl CoA Incorporated into Fatty Acids
	mμmoles
Complete system*	5.5
No ATP	0.1
No TPNH	2.0
No DPNH	1.8
No TPNH; no DPNH	0.0
Complete system + HCO_3^-	5.0

* The complete system contained 58.6 mμmoles 1-C^{14}-acetyl CoA (152,000 cpm), 2 μmoles ATP, 0.125 μmole DPNH, 0.125 μmole TPNH, 30 μmoles phosphate buffer (pH 6.5), and 1.0 mg of soluble extract (prepared by extracting the acetone powder of beef liver mitochondria with 10 volumes of 0.1 M phosphate buffer, pH 7.0, for 1 hour at 0°). Four μmoles $KHCO_3$ was added where indicated. The reaction mixture was incubated under N_2 at 38° for 1 hour.

efficient as a precursor of fatty acid synthesis than acetate. This system incorporated labeled acetate predominantly into fatty acids rather than into glycerides.

The pigeon liver system of Gurin and his collaborators was the basis for our studies on the mechanism of fatty acid synthesis (1, 2, 3, 5, 6, 7, 9, 10). This system can be prepared from chicken liver (67), rat liver, and rat kidney, as well as pigeon liver. When any of these tissues were extracted in a Potter-Elvehjem homogenizer with 0.1 M phosphate, 0.25 M sucrose, or 0.88 M sucrose and the extract fractionated into mitochondria, microsomes, and soluble fractions, the soluble fraction invariably contained all the enzymes required for the conversion of acetate or acetyl CoA to fatty acids (Table 4). The 100,000 × *g* supernatant

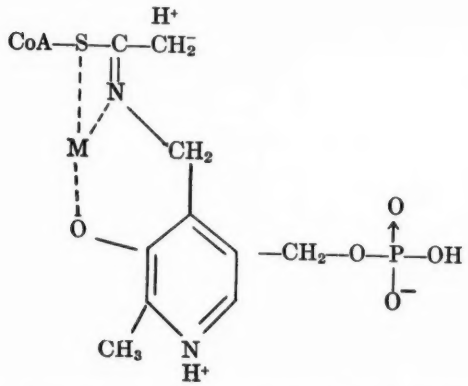


FIG. 4. A proposed model system for acetyl CoA, pyridoxamine phosphate, and metal (M).

TABLE 3. FACTORS AFFECTING FATTY ACID SYNTHESIS BY SOLUBLE MITOCHONDRIAL EXTRACTS

Exp.	Additions	Acetyl CoA Incorporated into Fatty Acids
		mμmoles
I	None	0.00
	ATP	0.29
	ATP and octanoic acid	3.00
	ATP and pyridoxal phosphate	0.79
	ATP and octanoic acid and pyridoxal phosphate	4.50
	ATP and octanoic acid and pyridoxamine phosphate	4.50
II	None	0.00
	ATP	0.20
	ATP and octanoic acid	1.88
	ATP and octanoic acid and pyridoxal phosphate	3.54
	ATP and octanoic acid and pyridoxamine phosphate	3.16
III	ATP	0.2
	ATP and pyridoxal phosphate	0.6
	ATP and boiled enzyme extract	1.3
	ATP and boiled enzyme extract and pyridoxal phosphate	2.61
	ATP and boiled enzyme extract and pyridoxamine phosphate	2.28
	ATP and boiled enzyme extract and pyridoxine HCl	1.5
	ATP and boiled enzyme extract and pyridoxamine HCl	1.88
IV	None	.08
	Palmityl CoA	1.30
	Palmityl CoA plus pyridoxal phosphate	2.00
V	Palmityl CoA plus ATP	1.0
	Palmityl CoA plus ATP and pyridoxal phosphate	1.6

The reaction mixture contained 30 μmoles of phosphate buffer (pH 6.5), 58 mμmoles of 1-C^{14}-acetyl CoA (152,000 cpm), 0.125 μmole DPNH, 0.125 μmole TPNH, and water to a final volume of 0.5 ml. Where indicated, the following were added: 2 μmoles ATP, 25 mμmoles octanoic acid, 50 mμmoles pyridoxal phosphate, 150 mμmoles pyridoxamine phosphate, 150 mμmoles pyridoxine HCl, 150 mμmoles pyridoxamine HCl, 10 mμmoles palmityl CoA, and 0.05 ml of boiled enzyme extract (prepared by boiling the soluble extract of mitochondria [cf. Table 2] for 5 minutes at 100°).

Five different dialyzed enzyme preparations were added as follows: In experiment I, 0.13 mg of charcoal-treated soluble extracts of beef liver mitochondria; in experiment II, 0.2 mg of charcoal-treated soluble extracts of beef liver mitochondria; in experiment III, 0.62 mg of soluble extract of beef liver mitochondria; in experiment IV, 0.55 mg of soluble extract after sonic oscillation of rat liver mitochondria, and in experiment V, 1.5 mg of mitochondria after sonic oscillation.

All tubes were incubated under N_2 gas for 1 hour at 38°.

fraction shows higher activity per mg of protein than the whole homogenate minus cell debris. This is probably because of the removal of the inactive particles (mitochondria and microsomes) from the mixture. The results in Table 4 also show that addition of mitochondria or microsomes decreased the activity of the supernatant fluid. Similar results were obtained when the tissues were homogenized in hypertonic sucrose solution (0.88 M), which minimizes the destruction of

the mitochondria during the homogenization. Furthermore, the type of fitting (loose or tight) of the homogenizer appeared to have very little effect on the extraction of the fatty acid synthesizing system from these tissues.

From these experiments it was concluded that the enzymes of fatty acid synthesis are localized in the soluble cytoplasmic portion of the pigeon and rat liver cells. A similar distribution of the fatty acid synthesizing system was found by Brady *et al.* in pigeon liver (68), by Langdon (17) in rat liver, and by Popják and Tietz (69, 70) in lactating mammary gland. Therefore, we would like to designate this pathway as the "non-mitochondrial system" in order to differentiate it from the mitochondrial system" discussed previously. Klein (71) studied the fatty acid synthesizing system from yeast (*S. cervisiae*) and reported that the conversion of acetate to long-chain fatty acids required both the soluble fraction plus what he called "small particle fraction" sedimented between 25,000 and 60,000 $\times$ *g*, free from the cytochromes and cytochrome oxidase). This also supports our conclusion that the fatty acid synthesizing system is a non-mitochondrial system in origin.

The fatty acid synthesizing system from pigeon liver, rat liver, or rat kidney can be separated from the 100,000 $\times$ *g* supernatant fluid by centrifugation of this extract at 140,000 $\times$ *g* for 2 to 4 hours (7). The resulting supernatant fluid was inactive while the red-colored pellets contained all the enzymes required for the conversion of acetate to fatty acids (cf. Table 4). The structural relationship of the various enzymes of the fatty acid synthesizing sequence in these pellets are not as yet understood and must await further experimentation.

Purification of the Non-mitochondrial Enzyme System. The soluble extracts of pigeon liver were fractionated with ammonium sulfate into two fractions by Wakil *et al.* (1). The first fraction (designated as R_1) is precipitated between 0% and 25% saturation of ammonium sulfate, and the second fraction (designated as R_2) is precipitated between 25% to 40% saturation. Both of these fractions are required for the conversion of acetyl CoA to fatty acids. Earlier work (2, 7) indicated a requirement for a third fraction (R_4), precipitating between 50% to 65% saturation of ammonium sulfate. This extra fraction was shown to contain acetate thiokinase and was no longer necessary when acetyl CoA was used as the starting substrate instead of acetate.

The two fractions R_1 and R_2 were further purified by adsorption on calcium phosphate gel and subsequent elution with phosphate buffer (1, 7) followed by chromatography on a cellulose column (9), according to the general procedure of Sober and Peterson (72). The final purified fractions obtained are referred to as R_{1gc} (derived from R_1 fraction) and R_{2gc} (derived from R_2). The over-all purification of the fatty acid synthesizing system amounts to more than 1,000-fold based on the original 100,000 $\times$ *g* supernatant extract. This is a minimum value, since it is difficult to give an exact value in a multi-enzyme system of this nature.

Fractions R_{1gc} and R_{2gc} are free of some of the various enzymes of the β-oxidation cycle: fatty acid activating enzyme (73), acetic thiokinase (74, 75), butyryl dehydrogenase (76), palmityl dehydrogenase (77, 78), the electron-transferring factor (79), and thiolase (80-82). They do contain, however, traces of enoyl hydrase (83, 84) and β-hydroxyacyl dehydrogenase (80, 85). The TPNH α,β-unsaturated acyl CoA reducing enzyme (17), which is a key enzyme in the synthesis of fatty acids by the reversal of the β-oxidation sequence (35), is absent from R_{1gc} and R_{2gc}. These findings, in addition to the studies on cellular distribu-

TABLE 4. INCORPORATION OF LABELED ACETATE INTO FATTY ACID BY PIGEON LIVER FRACTIONS

Fraction Tested*	C^{14}-acetate Incorporated/hour/mg of Protein
	mμmoles
Mitochondria	2.0
Microsomes	0.8
Clear supernatant	18.0
Mitochondria + microsomes	1.2
Mitochondria + clear supernatant	5.0
Microsomes + clear supernatant	8.0
Pellets	42.0

* The various fractions were prepared by homogenizing pigeon liver in 0.25 M sucrose with the Potter-Elvehjem homogenizer. The whole homogenate was centrifuged at 600 $\times$ *g* to remove the nuclei and the cell debris. The mitochondria and the microsomes were isolated by the usual centrifugal fractionation at 9000 $\times$ *g* and 100,000 $\times$ *g*, respectively. The resulting clear supernatant solution was used as indicated above. The pellets were obtained by further centrifuging the clear supernatant solution for 4 hours at 100,000 $\times$ *g*. Each reaction mixture contained 5.0 μmoles of acetate-1-C^{14} (300,000 cpm total activity), 50 μmoles of potassium phosphate buffer (pH 6.5), 4 μmoles of ATP, 0.3 μmole of Mn^{++}, 0.1 μmole of TPN^+, 8 μmoles of isocitrate, 0.04 μmole of CoA, 6.0 μmoles of cysteine, and 10 μmoles of $KHCO_3$. Final volume was 0.5 ml, and the reaction was started by the addition of 2.5 mg of protein of each fraction where indicated. The reaction mixture was incubated at 38° for 2 hours.

tion, further differentiate the non-mitochondrial system from the mitochondrial system.

Products of the Reaction. Considerable experimental evidence has been obtained which supports the concept that fatty acids are synthesized by successive head-to-tail condensations of two carbon units (47, 86). Much of this information has been derived from earlier studies with whole animals or tissue slices (47, 48, 49, 86). Brady and Gurin (14) separated the long-chain fatty acids ($>C_{10}$) that were synthesized by soluble preparations of pigeon liver, and on decarboxylation of these fatty acids (synthesized from acetate-1-C^{14}), the radioactivity in the carboxyl carbon atom (cpm per C) of the fatty acid was found to be only slightly above the specific activity of the fatty acid (cpm per C), which is to be expected from successive condensations of acetate units. Popják and Tietz (69, 70) separated and identified the products of fatty acid synthesis in mammary gland homogenates, and these were shown to be predominately long-chain fatty acids (C_{10} to C_{18}). Similar results were reported by Klein (71) in the fatty acid synthesizing system from yeast, and by Stumpf and Barber (4) utilizing extracts of avocado fruits.

The products arising from the conversion of acetate to fatty acids by the relatively crude avian liver fractions (R_1 and R_2) have been separated and identified by a variety of techniques (38, 39). The major components were palmitic acid (80%) with myristic and lauric acids constituting the remainder; short-chain acids (C_4 and C_{10}) did not accumulate under the reaction conditions employed (3).

Palmitic acid synthesized from acetate-1-C^{14} was isolated by dilution with unlabeled palmitic acid and recrystallized from various solvents to a constant specific activity. The resulting acid was decarboxylated by the Schmidt reaction as described by Phares (40), and the liberated CO_2 was found to contain twice the average specific radioactivity of the other carbon atoms of the palmitic acid, indicating a *de novo* synthesis of palmitic acid from acetate.

Components and Properties of the Non-mitochondrial System. As mentioned above, Gurin and his coworkers (14, 15, 16) showed that the synthesis of long-chain fatty acids from acetate by the soluble extract from pigeon liver was markedly stimulated by Mg^{++} and citrate. When this extract was treated with charcoal, the synthesis was stimulated by the addition of CoA, ATP, and DPN^+ in the presence of Mg^{++} and citrate. However, after dialysis of the extract, the activity declined almost completely and could not be restored by the addition of the above cofactors. These observations were confirmed by the investigators at the Enzyme Institute (1, 2, 3), where earlier studies had demonstrated the following cofactors to be required for the conversion of acetate to fatty acids by the reconstituted crude system: ATP, CoA, GSH, DPN^+, glucose-1-phosphate, isocitrate, TPN^+, lipoic acid, Mg^{++}, and Mn^{++}. Some of these factors dropped out as purification of the enzyme system proceeded, whereas several new requirements emerged (5, 6, 7).

The purified system of Wakil and Gibson (R_{1gc} and R_{2gc}) required ATP, Mn^{++}, HCO_3^-, and TPNH (Table 5) for optical conversion of acetyl CoA to long-chain

TABLE 5. COMPONENTS OF THE FATTY ACID SYNTHESIZING SYSTEM OF PIGEON LIVER

	Acetyl CoA Incorporated into Fatty Acid
	μmoles
Complete system*	0.394
No ATP	0.000
No TPNH	0.000
No Mn^{++}	0.030
No HCO_3^-	0.010
No R_{1g} (or R_{2g})	0.000

* Complete system: 50 μmoles histidine buffer (pH 6.5); 2 μmoles ATP; 0.6 μmole $MnCl_2$; 0.6 μmole acetyl CoA; 0.8 μmole TPNH; 8 μmoles $KHCO_3$; 1 mg of R_{1g}; and 0.8 mg R_{2g}. Time: 30 minutes at 38°. Final volume, 1 ml.

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fatty acids. Very little or no synthesis took place in the absence of any one of these factors. The additional components required in the crude system, such as glucose-1-phosphate and isocitrate, may have provided a source for both CO_2 and reduced pyridine nucleotides. Furthermore, isocitrate may play a role similar to other di- and tricarboxylic acids such as malonate, α-ketoglutarate, succinate, fumarate, citrate, etc. (1, 2, 69, 70), which stimulate the synthetic process without being incorporated partly or wholly into the products (7). The exact mechanism of this effect is not yet clear. One possible interpretation for the stimulatory action of these polycarboxylic acids may be the protection of the malonyl CoA (itself a dicarboxylic acid derivative of CoA) against enzymatic destruction, e.g., deacylation to free acid and CoA, or decarboxylation to acetyl CoA, etc.

Earlier assays of the synthesis of fatty acids from acetate were based exclusively on the use of radioisotopes (1, 2, 3). However, in the purified enzyme sys-

tem, an equally sensitive and far more convenient assay method was developed, based upon the spectrophotometrically measured rate of oxidation of TPNH by the fatty acid synthesizing system (87). Figure 5

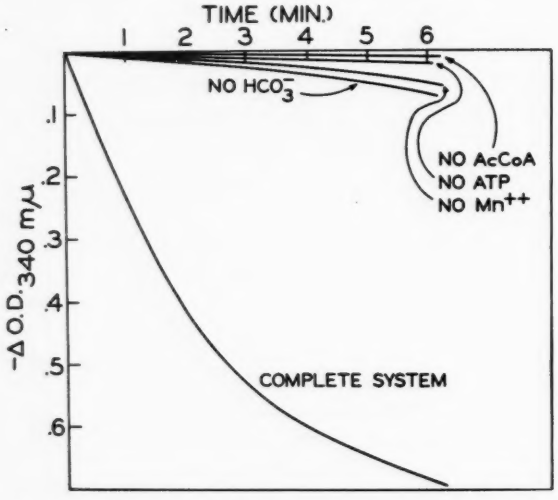

FIG. 5. Oxidation of TPNH. Each cuvette contained the following reagents (except when omitted as indicated): 25μmoles of potassium phosphate buffer (pH 6.5), 1.0 μmole of ATP, 0.3 μmole of $MnCl_2$, 4.0 μmoles of $KHCO_3$, 0.05 μmole of 1-C^{14}-acetyl CoA, and 0.08 μmole of TPNH in a final volume of 0.50 ml. The reaction was started by the addition of 0.7 mg of R_{2E^c}. The temperature was maintained at 38°. At the end of 5 minutes 0.032 μmole of acetyl CoA was incorporated into fatty acids.

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shows that the rate of oxidation of TPNH is strictly dependent upon the presence of all the components of the complete system. This rate parallels the rate of incorporation of C^{14}-1-acetyl CoA into the long-chain fatty acids as determined by the isolation of the fatty acids from the reaction mixture (1).

DPNH can substitute for TPNH in fatty acid synthesis, but the rate of oxidation of DPNH is about one-third the rate of oxidation of TPNH (87). This cannot be caused by the presence of DPNH-TPN^+ transhydrogenase (88), since we were unable to demonstrate transhydrogenase activity in the two enzyme preparations.

The lack of specificity for pyridine nucleotides distinguishes the non-mitochondrial system from the mitochondrial system (19), in which both TPNH and DPNH are required for the synthesis of fatty acids from acetyl CoA and fatty acyl CoA.

Acetyl CoA Carboxylase. The requirement of bicarbonate for fatty acid synthesis could be displayed by highly purified enzyme preparations obtained by Gibson *et al.* (5, 7). They demonstrated that this requirement was absolute for all stages of purification and that it applied to the synthesis of fatty acids not only in the avian liver preparation but also in crude preparations from rat liver and rat kidney. Figure 6

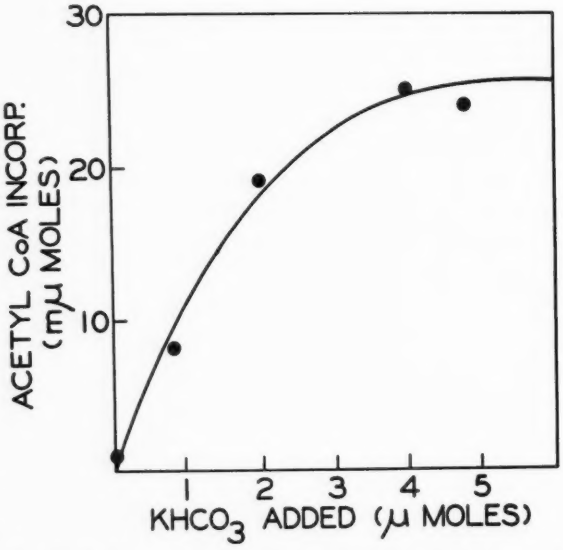

FIG. 6. Each experimental tube contained in a final volume of 0.5 ml the following reagents: 50 μmoles of potassium phosphate buffer (pH 6.5), 1.0 μmole of ATP, 0.3 μmole of $MnCl_2$, 0.08 μmole of TPNH, and 50 μmoles of acetyl-1-C^{14} CoA. The reaction was started by addition of 0.7 mg R_{1E} and 0.4 mg R_{2E}. All samples were incubated for 5 minutes at 38°. At a bicarbonate concentration of 8×10^{-3} M, the rate of acetyl CoA incorporation is equivalent to 0.3 μmole/mg enzyme/hour.

shows the effect of increasing concentration of bicarbonate on the synthesis of fatty acids in the purified system.

$HC^{14}O_3^-$ did not incorporate into the fatty acids during active synthesis from unlabeled acetyl CoA, as shown in Table 6. This is not surprising, since it had reason a "catalytic" role for HCO_3^- had been proposed (5, 7).

A similar requirement for bicarbonate was reported by Klein (71) in studies on the synthesis of fatty acids by particulate preparations from yeast cells, as well as by Stumpf and co-workers (8) in their studies on fatty acid synthesis in extracts from avocado fruit. This common requirement for fatty acid synthesis establishes the universality of the fatty acid synthesizing system in living cells.

Recently Wakil (9) was able to isolate the first intermediate in the synthesis of long-chain fatty acids from acetyl CoA and thus split the reaction sequence into two parts. When acetyl CoA was incubated with R_{1gc} in the presence of HCO_3^-, Mn^{++}, and ATP, an intermediate was isolated from the reaction mixture which could be converted to palmitate in the presence of R_{2gc} and TPNH. This intermediate incorporated both C^{14}-acetyl CoA and $HC^{14}O_3^-$ in a ratio of approximately 1:1. After hydrolysis with alkali, the saponifiable fraction was extracted with diethyl ether and was shown to contain all of the radioactivity. This compound was identified as malonate (9) by its R_f in two different chromatographic systems, melting point, and melting point of the *p*-nitrobenzyl ester. Formica and Brady (89) were also able to demonstrate the formation of malonyl CoA by the carboxylation of acetyl CoA by crude extracts of pigeon liver and pig heart.

The formation of the malonyl derivative by the purified enzyme preparation R_{1gc} is dependent on the presence of Mn^{++}, ATP, and HCO_3^- (Table 7). Thus it appears that ATP activates the HCO_3^- (or any of its equilibrium species, CO_2 and H_2CO_3), which is then condensed with the methyl group of acetyl CoA to form malonyl CoA. For this reason the name "acetyl CoA carboxylase" (referred to previously as R_{1gc}) has been suggested for this enzyme.

The product of the carboxylation reaction has been identified as malonyl CoA by paper chromatography in two different systems (isobutyric acid-ammonia and ethanol-sodium acetate). Its behavior was identical to that of chemically prepared malonyl CoA. This evidence led us to conclude that the first step in fatty acid synthesis is the formation of malonyl CoA from bicarbonate and acetyl CoA.

The reaction was absolutely dependent upon ATP for the activation of CO_2 prior to its condensation with acetyl CoA, with a simultaneous phosphorolysis of ATP into ADP and inorganic phosphate. The stoichiometry of the reaction showed that for each mole of malonyl CoA formed there was 1 mole of ATP utilized and 1 mole each of ADP and inorganic phosphate formed as shown in the following equation:

TABLE 6. FAILURE OF $H^{14}CO_3^-$ TO BECOME INCORPORATED INTO THE SYNTHESIZED FATTY ACIDS

Exp.	Complete System Plus*	TPNH (ΔA† 340 mμ)	Acetyl CoA Incorp. into F.A. (cpm)	$HC^{14}O_3^-$ Incorp. into F.A. (cpm)
I	Acetyl-1-C^{14} CoA and unlabeled HCO_3^-	0.460	8430	
	Unlabeled acetyl CoA and $HC^{14}O_3^-$ (6×10^5 cpm)	0.420		50
II	Acetyl-1-C^{14} CoA and unlabeled HCO_3^-	0.375	6800	
	Unlabeled acetyl CoA and $HC^{14}O_3^-$ (2×10^6 cpm)	0.370		0.00

* Each cuvette contained the following components of the complete system: 25 μmoles of potassium phosphate pH 6.5; 1 μmole ATP; 0.3 μmole of $McCl_2$; and 0.05 μmole of TPNH. As indicated, the following reagents were also added: 50 mμmoles of acetyl-1-C^{14} CoA (37,000 cpm) or 45 mμmoles of unlabeled acetyl CoA; 4 μmoles of either unlabeled HCO_3^- or $HC^{14}O_3^-$. Total volume was 0.5 ml. The reaction was started by the addition of 0.6 mg of R_{1g} and 0.5 mg R_{2g}.

† ΔA represents the change in absorbancy at 340 mμ, which was followed for 5 minutes at 30° in the Beckman DUR, and the amount of fatty acid synthesized was determined as usual at the end of this time.

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Our earlier reports on the stoichiometry of the overall conversion of acetyl CoA to palmitate employing relatively cruder fractions of R_{1g} and R_{2g} indicated that 2 moles of ATP were utilized for every mole of acetyl CoA converted to fatty acids (87). When the highly purified fraction R_{1gc} was used in the malonyl CoA formation, only 1 mole of ATP was consumed for every mole of acetyl CoA carboxylated to malonyl CoA. Brady (11) has recently reported on the possible formation of higher fatty acids (as identified by the R_f of their hydroxamic acids) from malonyl CoA by either crude R_1 or R_2 fractions. Furthermore, Lynen and his colleagues were able to confirm these observations, using purified enzyme preparations from yeast cells (12, 90).

$$CO_2 + CH_3\overset{\overset{O}{\|}}{C}SCoA + ATP \xrightarrow[(\text{bound-biotin}) + Mn^{++}]{\text{acetyl CoA carboxylase}} CH_2\begin{matrix}COOH \\ COSCoA\end{matrix} + ADP + P_i$$

TABLE 7. REQUIREMENTS OF ACETYL CoA CARBOXYLASE REACTION

	Malonyl CoA Formed
	mμmoles
Complete system*	12.0
No AcCoA	0.0
No ATP	0.0
No HCO_3^-	0.1
No Mn^{++}	0.1
No enzyme	0.0

* The complete system contained 20 mμmoles acetyl CoA, 1.0 mμmole ATP, 0.3 mμmole $MnCl_2$, 4 mμmoles $KHCO_3$, 30 mμmoles phosphate buffer (pH 6.5), and 0.100 mg of acetyl CoA carboxylase in a total volume of 0.4 ml. The reaction mixture was incubated for 10 minutes at 38° and the reaction was stopped by heat denaturation of the enzyme. Malonyl CoA was assayed by its oxidation of TPNH in the presence of the R_{2gc} enzyme fraction.

With the aid of malonyl CoA, Ganguly (91) was able to study the distribution of the non-mitochondrial system in various tissues. The results (Table 8) show that there is a wide distribution of this system in animal tissue, suggesting that this system is the main pathway for synthesis of fatty acids.

TABLE 8. DISTRIBUTION OF THE NON-MITOCHONDRIAL SYSTEM IN CRUDE EXTRACTS OF VARIOUS TISSUES

Tissue (Crude Extract)	Malonyl CoA Converted to Palmitate per mg of Protein per 10 Minutes
	mμmoles
Beef liver	0.15
Beef brain	1.8
Beef pancreas	0.3
Beef lung	0.23
Beef kidney	0.06
Beef small intestine	0.02
Beef mammary gland	5.3
Beef adipose tissue	2.3
Beef suprarenal fat	7.0
Beef aorta	0
Chicken liver	14.5
Chicken ovary	0.04
Chicken oviduct	0
Pigeon liver	28.9

Each assay tube contained 20 μmoles of potassium phosphate buffer (pH 6.5), 50 mμmoles of TPNH, 6 mμmoles (12,000 cpm total activity) of labeled malonyl CoA, limiting amount of the crude extract of the tissues indicated, and water to 0.40 ml. The The reaction mixture was incubated at 38° for 10 minutes.

The Role of Biotin in Fatty Acid Synthesis (Biotin in Acetyl CoA Carboxylase). Available information about the role of biotin in metabolic reactions points to a close relationship between this vitamin and the metabolism of carbon dioxide in the carboxylation-decarboxylation type reactions (for a comprehensive review see György (92)). A relationship has also been reported between biotin and fatty acid metabolism. Unsaturated fatty acids such as oleic, linoleic, etc., can promote growth of many microorganisms in the absence of biotin (93). Saturated fatty acids, although inactive alone, have a sparing effect on the unsaturated fatty acids. Until recently (6, 94) this relationship between biotin and fatty acid metabolism was very obscure and could not be related, on the surface at least, to the more generalized effect of biotin on the metabolism of carbon dioxide. The discovery by Gibson *et al.* (5, 7) that bicarbonate is an absolute requirement for fatty acid synthesis by a purified enzyme system suggested a possible role for biotin. When such enzyme fractions were assayed for biotin content by the procedure of Wright and Skeggs (95, 96), there was a significant concentration of this vitamin in one of the fractions, namely, R_{1gc} (acetyl CoA carboxylase) (6, 7, 94). Biotin concentrated with the active protein of this fraction all along the steps of purification (Fig. 7), and the ratio of enzymatic activity to biotin content remained essentially the same throughout the purification. The final concentration

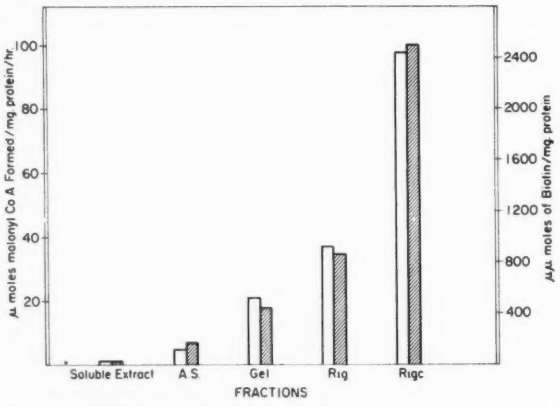

FIG. 7. The ratio of biotin content to specific activity of acetyl CoA carboxylase during the various steps of purification. The specific enzyme activity (μmoles acetyl CoA converted to malonyl CoA per mg protein per hour) of acetyl CoA carboxylase (left column) is compared with the protein-bound biotin concentration (right column).

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of biotin in the most purified preparation (after purification by ion exchange column) amounts to about 2 or 3 moles of biotin per 10^6 of protein or 1 mole of biotin per 300,000 to 500,000 of protein. This is the highest concentration of protein-bound biotin reported so far.

Biotin is tightly bound to the protetin and can be released only by tryptic digestion or acid hydrolysis (94). The product of tryptic digestion is not free biotin but a conjugated derivative of biotin, possibly biocytin (94), as shown by microbiologic assay (95) and paper chromatography (96). Free biotin is released by the acid hydrolysis of the protein fractions.

Wakil *et al.* (6, 94) have presented evidence to show that the protein-bound biotin does indeed participate in the over-all synthesis of long-chain fatty acids from acetyl CoA. They have shown that the conversion of acetyl CoA to palmitate is inhibited by avidin (92), an egg-white protein which specifically binds biotin (Fig. 8). This inhibition can be relieved by the addition of free *d*-biotin as shown in Figure 8. The effect of avidin can also be demonstrated spectrophotometrically as shown in Figure 9.

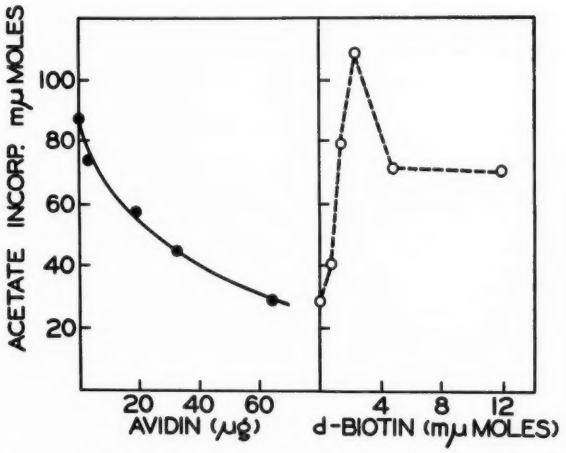

FIG. 8. Reversal of the avidin inhibition of fatty acid synthesis by D-biotin. In the left curve, increasing amounts of purified avidin are added to a series of identical incubation mixtures. The maximum inhibitory effect in this sequence was obtained with 64 μg avidin. The right curve demonstrates that by adding increasing quantities of free *d*-biotin to a second series of incubation mixtures, each containing 64 μg avidin, the inhibitory effect of avidin on synthesis is progressively eliminated. Each experimental system contained in a total volume of 0.5 ml the following reagents: 40 μmoles of phosphate buffer (pH 6.5); 0.3 μmole of $MnCl_2$; 6.0 μmoles of cysteine; 2.7 μmoles of ATP; 0.38 μmole of TPN; 6.0 μmoles of isocitrate; 0.04 μmole of CoASH; 1.32 μmoles of 1-C^{14}-acetate; 10 μmoles of $KHCO_3$; 0.3 mg acetic thiokinase (contains isocitric dehydrogenase); 0.9 mg R_{2g}. The reactions were initiated by the addition of 0.24 mg R_{1g}. Incubations were carried out at 38° for 30 minutes in an atmosphere of nitrogen.

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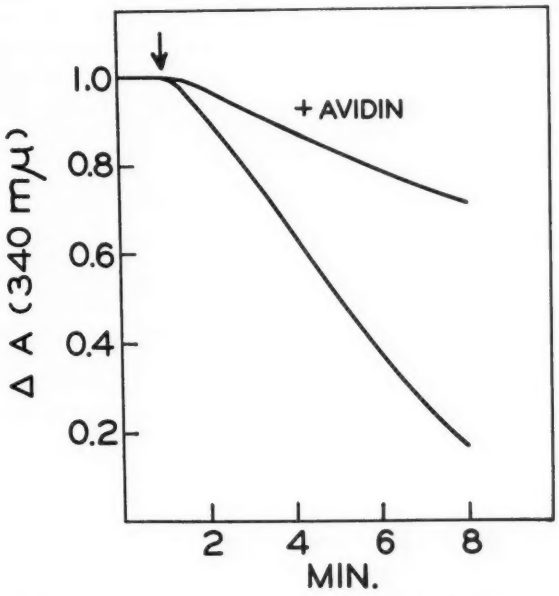

FIG. 9. Avidin inhibition of the oxidation of TPNH associated with fatty acid synthesis. Purified avidin (46 μg) was added to one of two identical systems each of which contained in a total volume of 0.50 ml the following reagents: 50 μmoles potassium phosphate buffer (pH 6.5); 0.3 μmole $MnCl_2$; 1.0 μmole ATP; 0.21 μmole acetyl CoA; 5.0 μmoles $KHCO_3$; 0.17 μmole TPNH; and 1.5 mg R_{2g}. The reaction was initiated in both systems by the addition of 0.26 mg R_{1g} (indicated by arrow). The optical density at 340 mμ (1.0 cm cell) was followed continuously in the Beckman DUR spectrophotometer (at 38°).

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Biotin-deficient animals (rats and chicks) were prepared[3] and their livers were used to prepare the fatty acid synthesizing system. The results show that the levels of acetyl CoA carboxylase in the livers of these animals is lower than the amount of carboxylase in the normal animals, but it is by no means absent. On purification of the enzyme from deficient animals, the activity of the enzyme increases, as does the concentration of biotin. At the highest purity level the biotin content of such enzyme preparations appears to be comparable to the preparations from normal animals. This observation indicates that biotin deficiency, however severe it may be, results in the decrease of the

[3] S. J. Wakil, D. M. Gibson, and A. E. Harper. Unpublished observations.

total level of the acetyl CoA carboxylase, but it does not completely eliminate this vital enzyme from the liver.

Lynen and his collaborators (12, 97) have recently isolated another biotin-containing enzyme which carboxylates β,β-dimethyl acrylyl CoA to form β-methyl glutaryl CoA. They presented evidence to show that biotin is an integral part of this enzyme, and that CO_2-biotin-enzyme is an intermediate in this reaction. The proposed scheme is as follows:

$$CO_2 + ATP + \text{biotin-enzyme} \longrightarrow CO_2 \sim \text{biotin-enzyme} + P_i + ADP$$

$$CO_2 \sim \text{biotin-enzyme} + \beta,\beta\text{-dimethyl acrylyl CoA} \longrightarrow \beta\text{-methylglutaryl CoA} + \text{biotin-enzyme}$$

A similar mechanism may be operative in the carboxylation of acetyl CoA to form malonyl CoA.

Conversion of Malonyl CoA to Palmityl CoA. Wakil and Ganguly (10) have shown that the second enzyme fraction (R_{2gc}) is able to convert malonyl CoA to palmityl CoA in the presence of acetyl CoA and TPNH. This conversion could be followed either spectrophotometrically by measuring the oxidation of TPNH, or isotopically by measuring the incorporation of C^{14}-labeled malonyl CoA into palmitate. The requirement for acetyl CoA in the conversion of malonyl CoA to palmitate is absolute, as shown in Figure 10.

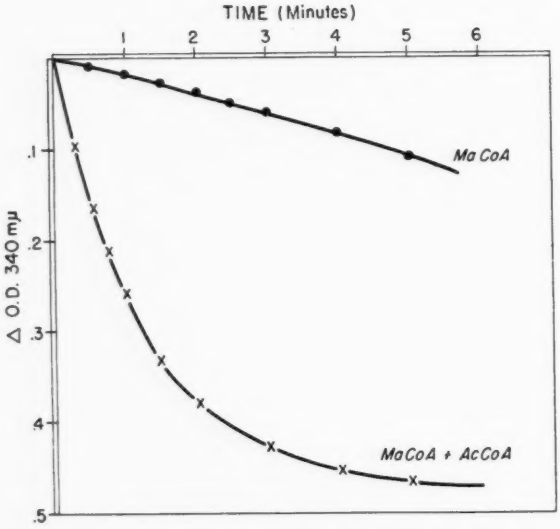


FIG. 10. The requirement for acetyl CoA in the oxidation of TPNH by malonyl CoA. Each cuvette contained 30 μmoles of phosphate buffer (pH 6.5), 0.015 μmole acetyl CoA where indicated, 0.016 μmole malonyl CoA, .05 μmole TPNH and water to a final volume of 0.4 ml. The reaction started with the addition of 0.2 mg of R_{2gc} fraction.

Furthermore, a significant amount of C^{14}-acetyl CoA was incorporated into palmitate when unlabeled malonyl CoA was used, as shown in Table 9.

The tendency of the reaction to proceed toward palmitate synthesis after incubation of malonyl CoA with enzyme in absence of acetyl CoA (Fig. 10) may result from the presence of a contaminating enzyme in the R_{2gc} preparation which decarboxylates malonyl CoA to acetyl CoA plus CO_2. Indeed, such an enzyme could be demonstrated in the cruder fractions of R_{2gc} by the formation of citrate from malonyl CoA in the presence of oxalo-acetate and Ochoa's condensing enzyme (98).

The data in Table 9 also show the stoichiometric relationship (based on 1 mole of palmityl CoA formed) between acetyl CoA, malonyl CoA, and TPNH in the synthetic process. The results show that for each mole of palmityl CoA synthesized, 1 mole of

TABLE 9. STOICHIOMETRY OF THE PALMITYL CoA FORMATION FROM MALONYL CoA PLUS ACETYL CoA

Acetyl CoA	Malonyl CoA	TPNH	Palmitate	CO_2	CoASH
mμmoles	*mμmoles*	*mμmoles*	*mμmoles*	*mμmoles*	*mμmoles*
	−8.2	−16.7	+1.1		+7.9
	−6.0	−12.3		+5.8	
−2.2		−25.8	+1.9		

Three separate experiments were used for the determination of the stoichiometry of the reaction. In the first experiment the reaction mixture contained 8.5 mμmoles C^{14}-malonyl CoA ($HOOCCH_2C^{14}OCoA$, 20,000 cpm), 50 mμmoles TPNH, 13 mμmoles acetyl CoA, 30 μmoles phosphate buffer (pH 6.5), 0.120 mg of R_{2gc}, and water to a final volume of 0.4 ml. The oxidation of TPNH was followed spectrophotometrically (cf. Fig. 10). At the end of the reaction (5 minutes, at 38°), aliquots were withdrawn for (a) CoASH analysis by the procedure of Wakil and Hübscher (105), (b) palmitate analysis by C^{14}-incorporation, and (c) malonyl CoA as determined by the amount of C^{14} left in the reaction mixture after extraction of C^{14}-palmitate.

In the second experiment the reaction mixture contained exactly the same reagents as in the first except that 6.0 mμmoles carboxyl labeled malonyl CoA ($HOOC^{14}CH_2COCoA$, 13,000 cpm) were used. The reaction was followed by TPNH oxidation. The $C^{14}O_2$ was trapped in NaOH and the radioactivity was determined as $BaC^{14}O_3$. Palmitate did not incorporate any C^{14}.

In the third experiment the reaction mixture was the same as in the first except that 13 mμmoles 1-C^{14}-acetyl CoA (32,000 cpm) and 30 mμmoles of unlabeled malonyl CoA were used. The remaining acetyl CoA was determined by both radioactivity and by the citrate condensing enzyme.

acetyl CoA, 7 moles of malonyl CoA, and 14 moles of TPNH are consumed and 7 moles each of CO_2, CoA, and water were formed. The over-all reaction can be presented as follows:

$$CH_3\overset{O}{\overset{\|}{C}}SCoA + 7HOOCCH_2\overset{O}{\overset{\|}{C}}SCoA + 14\,TPNH + 14H^+ \longrightarrow$$

$$CH_3(CH_2)_{14}\overset{O}{\overset{\|}{C}}SCoA + 14\,TPN^+ + 7CO_2 + 7CoASH + 7H_2O$$

In short, one "C_2-unit" of palmitate is derived from acetyl CoA, and the remaining 14 carbon atoms are derived from malonyl CoA.

Acetyl CoA contributes carbons 15 and 16 of palmitate as shown by degradation studies of the carboxyl group of palmitic acid derived from 1-C^{14}-acetyl CoA and 1-C^{14}-malonyl CoA, respectively. The carboxyl group of palmitic acid derived from 1-C^{14}-acetyl CoA had no radioactivity, while the carboxyl group of palmitic acid synthesized from malonyl CoA ($HOOCCH_2C^{14}OSCoA$) had the same specific activity as that of the labeled carbonyl group of malonyl CoA.

Acetaldehyde does not substitute for acetyl CoA under these conditions (10) nor does it dilute the amount of C^{14}-acetyl CoA incorporated into palmitate. These results are contrary to Brady's proposed mechanism for fatty acid synthesis (11), which implicated acetaldehyde as an intermediate.

Propionyl CoA can substitute for acetyl CoA, but other acyl CoA's, such as hexanoyl or octanoyl CoA, which do not incorporate into palmitate, cannot. 1-C^{14}-Butyryl CoA is incorporated into palmitate to an extremely low extent, as shown in Figure 11. This is contrary to our earlier reports, based on experiments utilizing cruder enzyme preparations, that butyryl CoA or octanoyl CoA (10 can be substituted for acetyl CoA. The pathway of incorporation of butyryl CoA or octanoyl CoA into palmitate in these experiments is not yet clear; nevertheless, with highly purified subfractions of R_2 only acetyl CoA or propionyl CoA can be incorporated into long-chain fatty acids, as shown in Figure 11.

When the rate of palmitate formation, as measured by the rate of oxidation of TPNH, was plotted against the concentration of acetyl CoA or propionyl CoA, the usual hyperbolic plot was obtained (Fig. 12). The same data could be plotted by the method of Lineweaver and Burk (Fig. 13), yielding two straight lines which intercept the abscissa at a single point. From such plots the Michaelis-Menten constants for acetyl CoA, propionyl CoA, and butyryl CoA were determined and found to be 2.3×10^{-6} M, 5.7×10^{-5} M, and 3.9×10^{-4} M, respectively. These values indicate that the affinity of the enzyme for propionyl CoA is about one twenty-fifth that of acetyl CoA, whereas with butyryl CoA the value is one one-hundred-and-seventieth that of acetyl CoA (Fig. 12).

When propionyl CoA was used instead of acetyl CoA, the product of the synthesis was an odd-chain fatty acid with 17 carbon atoms, indicated by the slightly lower R_f of this acid as compared to palmitate. This observation, therefore, may explain the occurrence of odd-chain fatty acids in animal tissues. This appears to be dependent on the availability of propionyl CoA to the cell rather than on the presence of another enzyme system for the synthesis of odd-chain fatty acids.

Incorporation of Tritiated Acetyl and Malonyl CoA into Palmitate.[4] In 1937 Sonderhoff and Thomas (42) demonstrated for the first time that deuterium-labeled acetic acid (CD_3COOH) could be incorporated into long-chain fatty acids by yeast cells. These results were confirmed by Barker *et al.* in 1945, and were extended by Rittenberg and Bloch to include experiments on whole animals (rats and mice) in which they showed that deuterium-labeled acetate incorporated in cholesterol as well as fatty acids. We recently studied the incorporation of tritium-labeled acetyl CoA ($CT_3COSCoA$) and tritium-labeled malonyl CoA ($HOOCCT_2COSCoA$) into palmitate by the purified enzyme preparation (R_{2gc}).[5] We hoped that such studies might help us formulate and understand the mechanism of fatty acid synthesis.

Tritium-labeled acetyl CoA was prepared enzymatically from tritiated acetate (CT_3COOH) and the acetic thiokinase system (74, 75). The tritiated acetyl CoA was then used to prepare the tritium-labeled malonyl CoA ($HOOCCT_2COSCoA$) employing the acetyl CoA carboxylase system. When tritium-labeled acetyl CoA ($CT_3COSCoA$) was added to the reaction

[4] This section dealing with tritiated substrates was received for publication on November 2, 1960.

[5] S. J. Wakil and R. Bressler. Unpublished results.

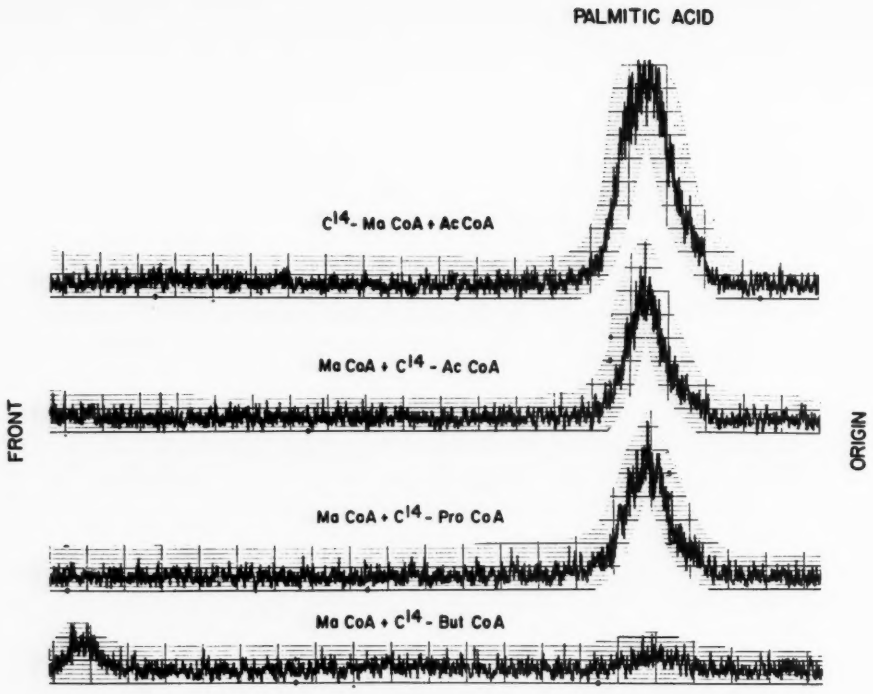


FIG. 11. Scanning of the paper chromatograms of the fatty acids synthesized from various labeled substrates. Four separate tubes contained 30 μmoles phosphate buffer (pH 6.5) and .05 μmole TPNH in a final volume of 0.4 ml. The following were also added: To the first tube, 0.01 μmole C^{14}-malonyl CoA ($HOOCCH_2C^{14}OSCoA$, 18,000 cpm) and 0.01 μmole acetyl CoA; to the second tube, 0.01 μmole of malonyl CoA and 0.013 μmole 1-C^{14}-acetyl CoA (32,000 cpm); to the third tube, 0.01 μmole malonyl CoA and 0.065 μmole 1-C^{14}-propionyl CoA (135,000 cpm); to the fourth tube, 0.01 μmole malonyl CoA and 0.08 μmole 1-C^{14}-butyryl CoA (170,000 cpm). The reaction was started by the addition of 0.180 mg of protein (R_{2gc}) and incubated at 38° for 5 minutes. The fatty acids were extracted in pentane after hydrolysis of the acyl CoA derivatives (1) and were chromatographed in the reverse phase system of Kaufmann and Nitsch (39). "Palmitic acid" is the only acid formed. The extra peak in the experiment of C^{14}-butyryl CoA is due to C^{14}-butyric acid.

mixture containing nonlabeled malonyl CoA, TPNH, and the R_{2gc} enzyme, tritium-labeled fatty acids were isolated. The incorporation of tritium from acetyl CoA was absolutely dependent upon the presence of malonyl CoA. The amount of tritium incorporated into the palmitate corresponded to about 1 mole of acetyl CoA incorporated into 1 mole of palmitate (as measured by TPNH oxidation), or 3 atoms of tritium were incorporated per molecule of palmitate. This is in complete agreement with the formulation given above for the synthesis of palmityl CoA from acetyl CoA and malonyl CoA. It would also indicate that the methyl group (CT_3) of acetyl CoA is incorporated into palmitate as a unit (presumably as carbon 16 of palmitate) with no loss of tritium atoms during this transformation.

When tritium-labeled malonyl CoA ($HOOCCT_2$-COSCoA) was added to nonlabeled acetyl CoA, TPNH, and the R_{2gc} enzyme, tritium-labeled palmitic acid was also isolated. The amount of tritium labeling in palmitic acid in three such experiments amounted to about 5 to 6.8 microatoms of tritium per μmole of palmitic acid synthesized. Since our stoichiometric formulation for palmitic acid synthesis indicated the consumption of 7 moles of malonyl CoA for each mole of palmityl CoA formed, then it is reasonable to conclude that there was approximately 1 atom of tritium incorporated into palmitate per "C_2-unit" derived from malonyl CoA. In other words, of two possible tritium atoms that might incorporate into palmitate from $HOOCCT_2COSCoA$, only one tritium atom was found in palmitate, while the other was presumably lost to the medium in the form of water (THO) in the course of the reaction.

These results did not lend support to our earlier suggestion that dicarboxylic acyl CoA's may be involved as intermediates in the conversion of acetyl CoA and malonyl CoA to palmitate. If the latter were the case, then we could not expect any incorporation of tritium atoms from malonyl CoA into palmitate. Therefore a modification of the scheme of Wakil and Ganguly must be made in order to take these observations into account. Such a modification must assume that the release of CO_2 from malonyl CoA takes place prior to the reduction of the carboxyl group, elimination of H_2O, and the reaction of the carbon-carbon double bond.

A likely possibility is that the condensation and decarboxylation of malonyl CoA may take place simultaneously. It is also conceivable that a negatively charged hypothetical intermediate such as this ($^-CT_2COSCoA$) may be formed on the enzyme surface as a result of the interaction of the enzyme with malonyl CoA. Such intermediate is then coupled with the partially positive charged carbonyl group of acetyl CoA ($CH_3-\overset{O^-}{\overset{|}{C^+}}-SCoA$) to form the keto derivative according to the following formulation:

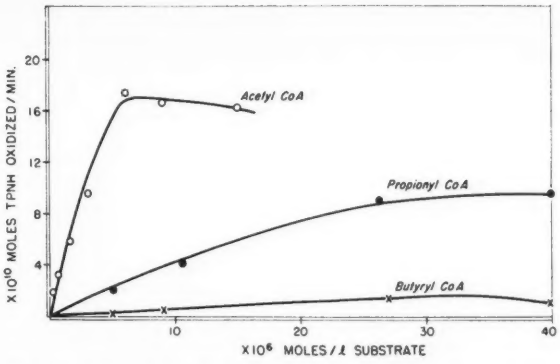
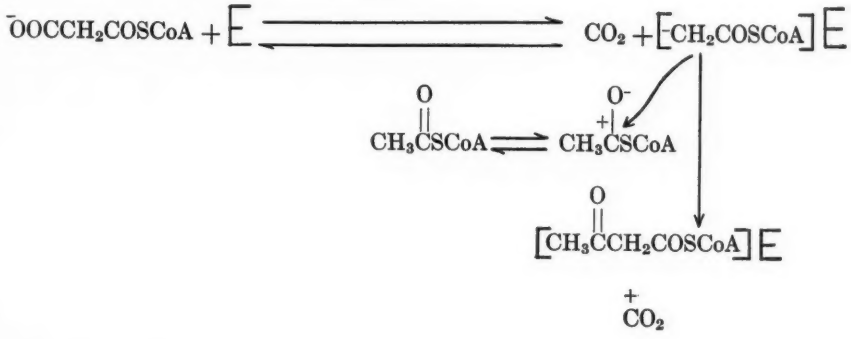


FIG. 12. The relationships between the rate of fatty acid synthesis (as measured by the rate of TPNH oxidation) and acetyl CoA, propionyl CoA, or butyryl CoA concentrations. Each reaction mixture contained 30 μmoles of phosphate buffer (pH 6.5), 0.016 μmole malonyl CoA, .05 μmole TPNH, the indicated amounts of acetyl CoA, propionyl CoA or butyryl CoA, and water to a final volume of 0.4 ml. The reactions were started by the addition of 0.1 mg of R_{2gc}. Temperature was 38°. The rate of the reaction was linear for the first 3 minutes.

Other possibilities cannot be ruled out at the present. Some of these may be the formation of polyketo acyl CoA (12) or an acyl derivative of a coenzyme other than CoA.



Very little is known about the nature of the β-keto derivatives. It probably is not acetoacetyl CoA, since the latter compound does not oxidize TPNH in the presence of the enzyme R_{2gc}; neither does it incorporate into palmitate. Lynen (90) has suggested that it is an acyl derivative of the enzyme

$$(CH_3-\overset{O}{\overset{\|}{C}}-CH_2-\overset{O}{\overset{\|}{C}}-SE)$$

which can be formed as a result of the condensation of acetyl CoA and malonyl-S-enzyme, but it is not formed by the interaction of acetoacetyl CoA and the enzyme.

The loss, as water, of one of the tritium atoms of the α-carbon of malonyl CoA during the synthesis of palmitic acid is of extreme interest and represents the most direct evidence so far for the dehydration of the intermediate(s) during the sequential transformation of malonyl CoA to fatty acids.

The elimination of water would undoubtedly yield an α,β-unsaturated acyl compound which is then reduced by TPNH to form the saturated derivative. It is too early as yet to say whether the reduction of (>C=C<) by TPNH takes place by direct transfer

of electrons from the pyridinenucleotide or through the intermediate oxido-reduction of another coenzyme (such as flavins in flavoproteins). It is interesting to note in this regard that all the known oxido-reduction reactions of DPN^+ and TPN^+ involve substrates with substituted carbons (e.g, >CHOH, $>CHNH_2$, etc.) and none that are >CH—CH<. On the other hand, there are numerous examples where flavoproteins are involved in the oxido-reduction of the carbon-carbon bond.

The Possible Mechanisms of Fatty Acids Synthesis. In a preliminary communication (10) we proposed a scheme for palmityl CoA synthesis from acetyl CoA and malonyl CoA which at the time appeared to explain our observations. This hypothesis proposed the formation of a C_5 intermediate (possibly acetomalonyl CoA) as the compound formed from the condensation of acetyl CoA and malonyl CoA. This C_5 compound was then thought to be successively reduced, dehydrated, reduced again, and decarboxylated to yield butyryl CoA. Thus in five successive steps, butyryl CoA was thought to be formed from acetyl CoA plus malonyl CoA. The butyryl CoA could condense with malonyl CoA to yield a C_7 dicarboxylic acyl CoA which would undergo the same successive transformations to yield a C_6 CoA, and the process would be repeated until finally palmityl CoA was formed. This hypothesis was based primarily on the ability of butyryl CoA and octanoyl CoA to be incorporated into palmitate (experiments performed with relatively crude enzyme preparations) and the inability of acetoacetyl CoA, β-hydroxyacyl CoA, and crotonyl CoA to oxidize TPNH and be incorporated into palmitate.

Lynen (90) subsequently offered another proposal, embodying the formation of tightly bound acyl-S-enzyme as an intermediate in order to explain the aforementioned observations. Lynen's scheme assumed that malonyl-S-enzyme is first formed from malonyl CoA and enzyme-SH, which is then condensed with an acyl CoA (acetyl CoA, butyryl CoA, etc.) and simultaneously decarboxylated to form a β-ketoacyl-S-enzyme. The latter derivative would be reduced, dehydrated, and reduced again to form the saturated acyl-S-enzyme which would then undergo an ester interchange reaction with CoASH to yield acyl CoA and free HS-enzyme. This scheme assumed the presence of only one enzyme responsible for the entire sequence, and required that the saturated acyl CoA's (C_4CoA, C_6CoA, etc.) were intermediates in the synthesis of palmityl CoA.

Our recent experiments on the mechanism of fatty acid synthesis employing highly purified enzyme preparations from pigeon liver appear to be somewhat different from those with crude enzyme preparations. The new observations can be summarized as follows: (*a*) Only acetyl CoA and propionyl CoA can be incorporated into long-chain fatty acids in the presence of malonyl CoA, TPNH, and enzyme. (*b*) One mole of acetyl CoA or propionyl CoA is incorporated into 1 mole of the fatty acid and constitutes the last 2 or 3 carbons, respectively, of the synthesized C_{16}- or C_{17}-fatty acid. (*c*) Butyryl CoA substitutes for acetyl CoA only to a very small extent (Figs. 11, 12), whereas hexanoyl CoA, octanoyl CoA, etc., do not incorporate at all into "palmitate." (*d*) With highly purified enzyme there is only one fatty acid synthesized from acetyl CoA plus malonyl CoA or propionyl CoA plus malonyl CoA, namely, C_{16}- or C_{17}-acids, respectively (Fig. 11). (*e*) Experiments (including trapping and dilution techniques) designed to isolate short-chain acyl CoA's (butyryl CoA, hexanoyl CoA, etc.) from the reaction mixture were not successful. (*f*) We have been unable to isolate the first condensation product (C_5 CoA) or any other dicarboxylic acyl CoA. Steberl *et al.* (99) reported the isolation of a "C_5" and "C_7" dicarboxylic

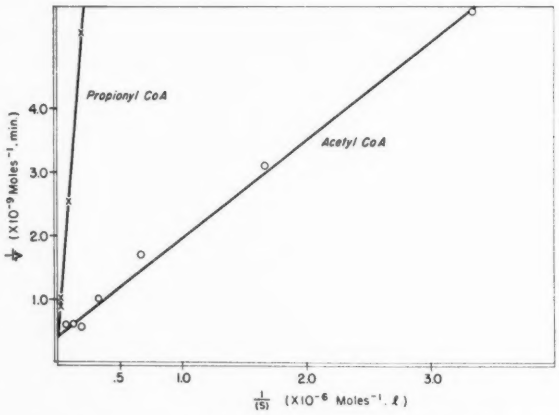


FIG. 13. Lineweaver and Burk plot of the data in Fig 12. for acetyl CoA and propionyl CoA.

acyl CoA from the condensation of acetyl CoA and butyryl CoA with malonyl CoA, respectively. However, the exact structures of these intermediates and their role in fatty acid synthesis must await additional information. (*g*) Substituted acyl CoA's (acetoacetyl CoA, β-hydroxybutyryl CoA, crotonyl CoA) do not oxidize TPNH in the presence of purified enzyme. They do not incorporate into the fatty acid nor do they dilute the amount of C^{14}-malonyl CoA converted to palmitate. (*h*) For each mole of palmityl CoA synthesized, 1 mole of acetyl CoA, 7 moles of malonyl CoA,

and 14 moles of TPNH were consumed and 7 moles of CO_2, 7 moles of CoASH, 14 moles of TPN, and 7 moles of water were formed. (*i*) Three tritium atoms from tritium-labeled acetyl CoA ($CT_3COSCoA$) were incorporated into 1 molecule of palmitate, indicating that the α-carbon of acetyl CoA constituted carbon 16 of palmitate. (*j*) One tritium atom of the tritium-labeled malonyl CoA $HOOCCT_2COSCoA$ was incorporated into palmitate per one "C_2-unit" derived from malonyl CoA, while the other tritium atom was presumably lost as water during the reactions.

These observations do not lend complete support to either Wakil and Ganguly's hypothesis based on a reaction sequence similar to β-oxidation and involving dicarboxylic acyl CoA derivatives, nor Lynen's acyl-S-enzyme hypothesis, which requires a multicatalytic enzyme which is without precedent in such varied and cyclic reactions.

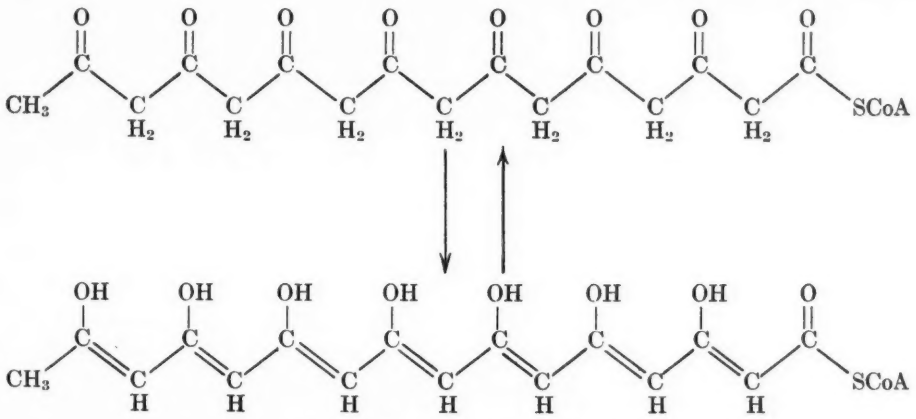


FIG. 14. Poly-keto palmityl CoA and its enol-form as one of the suggested intermediates in synthesis of palmityl CoA.

Further information is necessary in order to substantiate this mechanism. One of the attractive hypotheses is that proposed earlier by Lynen and later dropped in favor of the above. This involves the formation of a polyketo polymer of C_2-units (polyketo palmityl CoA; Fig. 14), which is then reduced completely to palmityl CoA. Such a compound might be formed by the condensation of 1 mole of acetyl CoA and 7 moles of malonyl CoA with concomitant formation of 7 moles of CO_2 and CoASH. The polyketo palmityl CoA formed could be reduced by TPNH to palmityl CoA. Although it is very difficult to conceive of the complete enzymic reduction of the polyketo acid occurring in a single step, it is conceivable that such a reduction of the undoubtedly predominant enol form may take place with the simultaneous removal of water, provided that during the tautomeric shifts there were no loss of tritium from the methyl group of the polyketo acid.

Mammary Gland System. Popják and Tietz (69, 70, 100) have studied fatty acid synthesis in homogenates and in soluble preparations from the mammary gland of lactating rats. The soluble supernatant fraction shows greater activity in synthesizing fatty acids from acetate than does the full homogenate. Neither the mitochondrial nor the microsomal fractions were required for fatty acid synthesis. ATP was absolutely required for synthesis, and when the enzyme preparation was treated with Dowex-1 resin, a requirement for CoA and DPN was noted. Oxalacetate, α-ketoglutarate, and succinate markedly stimulated the synthesis. Malonate stimulated the synthesis of fatty acids markedly, and in combination with β-ketoglutarate it gave as much as a thirtyfold increase in the amount of C^{14}-acetate incorporated into the fatty acids. The mammary gland extract synthesizes both short (C_4 to C_8) and long-chain fatty acids (C_{12} to C_{18}), while the whole homogenate preparation synthesizes predominantly long-chain fatty acids (1).

Hele *et al.* (101) prepared a fatty acid synthesizing system from the mammary glands of lactating rabbits which had essentially the same characteristics as previous systems from rat and sheep. On fractionation with ammonium sulfate (35% to 65%) an enzyme preparation was obtained which synthesized short-chain fatty acids from acetate in the presence of ATP, CoA, Mg^{++}, cysteine, and DPNH. They were unable to demonstrate any requirement for TPN^+ or for TPNH. Furthermore, the authors (101, 102) found that predominantly short-chain fatty acids were synthesized by the ammonium sulfate fractions of the mammary gland. According to the data of Hele (102),

there was much less octanoate and hexanoate synthesized than β-hydroxy octanoate. In other words, the amounts of saturated fatty acids formed, except for butyrate, were extremely small compared to the β-hydroxy and unsaturated acids. It is of great interest to note that in crude homogenates Popják and Tietz's description (69, 70, 100) of the fatty acid synthesizing system of mammary gland is remarkably similar to that of the pigeon, rat and chicken liver system described earlier. It may be possible that the mammary gland has two soluble fatty acid synthesizing systems, one for the synthesis of the short-chain acids (the system described by Hele and Popják (101)) and the other, the non-mitochondrial system similar to that described by Wakil *et al.* (1).

Further information is needed in order to ascertain this point.

SUMMARY AND CONCLUSION

Evidence has been presented to show that there are two distinct systems for the synthesis of fatty acids.

1. The mitochondrial system, which may involve some enzymes of the β-oxidation system (enoyl hydrase, β-hydroxyacyl dehydrogenase) working in reverse plus the TPNH-α, β-unsaturated fatty acyl CoA reductase and perhaps a new condensing enzyme. Both TPNH and DPNH are required for the synthesis. Essentially this system is for the elongation of the existing fatty acids by the addition of two-carbon units at a time. It is possible that this system may be responsible for the formation of stearate from palmitate, arachidonate from linoleate, etc.

2. The non-mitochondrial system, which is located in the cytoplasm of the cell and catalyzes the conversion of acetyl CoA to palmitate in the presence of ATP, Mn^{++}, HCO_3^-, and TPNH (DPNH may be substituted for TPNH with a slower rate). Acetyl CoA is condensed with HCO_3^- to form malonyl CoA in a reaction catalyzed by acetyl CoA carboxylase (a biotin-containing enzyme) in the presence of ATP and Mn^{++}. The biotin is bound to the protein, and evidence has been presented to show that it does participate in the formation of malonyl CoA. Malonyl CoA condenses with acetyl CoA or propionyl CoA and yields, on reduction by TPNH, saturated fatty acids, CO_2, and CoA.

This system appears to be the main pathway for fatty acid synthesis and is widely distributed in living organisms. So far this system has been isolated from pigeon liver, chicken liver, rat liver, rat kidney, yeast cells, and avocado fruits.

The enzymatic system for the synthesis of short-chain fatty acids in mammary gland may be different from the above two systems, but final judgment has to await more information.

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Volume 2
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Review

Structure and specificity of the lipid haptens of animal cells*

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The capacity of proteins and polysaccharides to evoke an antibody response is well established, and there is little difficulty in understanding the structural basis for the reactions of these antigens with specific antibody. In marked contrast, the role of lipids as antigens is still rather ill-defined. Although it is clear that a lipid molecule (cardiolipin) can serve as a determinant of immunological specificity, the number of examples is insufficient to support the formulation of generalizations relating the structure of lipids to their immunological activity. The recent isolation (1, 2) of a second pure lipid with antigenic specificity (cytolipin H) has provided the first model with exactly defined structure, and has demonstrated that certain distinctive features are characteristic of the immunological reactivity of lipids. Since this information permits studies of this type to be pursued systematically, it is worthwhile to review the problem at this time, despite the meagerness of the information at hand. This review will therefore place much greater emphasis on general principles that are important for the immunological study of lipids than on presenting a complete survey of the relevant literature.

Lipids with immunological activity belong to a larger group of cellular substances that have been designated as haptens since they react with antibodies, although they are ineffective in evoking antibody response. It would be difficult to improve on Landsteiner's succinct summary (3*a*) of the state of knowledge concerning cellular haptens despite the passage of more than fifteen years, and, indeed, the far-reaching significance of these substances in animal biology has had no greater champion. It was Landsteiner's view (3*b*) that "there exist two systems of species specificity in the animal kingdom, the specificity of proteins and that of cell haptens. The proteins . . . undergo gradual variation in the course of evolution, while haptens are subject to sudden changes not linked by intermediary stages." The immunological evidence for this point of view lay in "the striking serological differences between the cells of individuals of the same species, the frequent occurrences of so-called heterogenetic antigens, i.e., similarly reacting substances present in unrelated kinds of organisms, and the fact that blood cells of closely related species exhibit . . . much greater differences than the respective serum proteins." The nonprotein nature of cell haptens gave added weight to this thesis. Landsteiner was sensitive to the need for a chemical definition of antigenic structure that would lay a firm foundation for immunological application. It is, of course, not difficult to appreciate the reasons for the lag between chemical and serological analysis of cells, and it is a matter of record that in the past decade at least a dozen new antigenic determinants have been located in the human erythrocyte mosaic without the structure of a single one, new or old, being established. The principal difficulty resides in the fact that these substances are present in low concentrations in animal cells, and major technical problems are associated with separating trace substances from complex mixtures. A second obstacle stems from the fact that a serological method that may be perfectly adequate for investigation of whole cells may prove to be insensitive or otherwise unsuit-

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able when applied to the artifact combinations of substances that result once chemical purification is undertaken.

LIPID HAPTENS: OPERATIONAL DEFINITION

The methodological limitations in studies of haptens are particularly evident when they belong to the class of substances extractable from tissues with nonaqueous solvents. In studies of such substances, ethanol has been the solvent most frequently employed, and extractability with ethanol has been the usual criterion for classifying them as lipids (lipoid, lipid-soluble). This criterion may eventually prove to be reasonably sound, but it is not rigorous; and because nonlipid material may also be extracted, it does not permit the ultimate simplification that is required when mixtures of antigens must be resolved using reagents that are mixtures of antibodies. Antiserum reagents are usually obtained by hyperimmunization (repeated injection) of an animal with foreign cellular material, and antisera specific for a single antigen (or hapten) are not obtained.

One of the principal technical advantages of working with lipids is that the number of immunologically active lipids in a given cell appears to be small, and the water-soluble substances (proteins, polysaccharides) representing most of the antigenic complex can be rapidly eliminated. The introduction by Folch *et al.* (4) of chloroform-methanol extraction of tissues, and the ingenious method for efficiently washing this extract with water, represented a notable advance in the approach to a rigorous definition of the lipid nature of cell haptens by simple, reproducible means. In a comparative study of chloroform-methanol versus alcohol extraction of human tissues, it was found[1] that, with occasional exceptions, both methods gave results in qualitative agreement, although higher titers against antisera were usually obtained with chloroform-methanol extracts. Removal of water-soluble material, for which a convenient procedure is not available for ethanol extracts, will sometimes eliminate most of the reactive substance present in a chloroform-methanol extract, and may be useful in differentiating some haptens. It is clear that these techniques do not provide an entirely satisfactory answer to the question of what constitutes a "lipid" hapten, since a complex molecule may not always be readily classified by such procedures. The achievement here is mainly in the assurance that an active substance which remains consistently in the organic solvent phase will respond to chemical fractionation methods that are useful for lipids in general.

[1] L. Graf and M. M. Rapport, unpublished observations.

MEASUREMENT OF IMMUNOCHEMICAL ACTIVITY OF LIPID HAPTENS

Having defined a "lipid" hapten in operational terms, the next problem to be discussed is their measurement by immunochemical techniques. Several methods are available and have, in fact, been employed by various workers: flocculation, precipitation, complement-fixation, inhibition of cell agglutination or lysis, and, most recently, a combination of precipitation and paper chromatography (5). These methods are not equally serviceable for all purposes. It should be kept in mind that in most immunological studies, antibody estimation has been the goal, and once a suitable formulation of the "test antigen" was achieved, further variations were unnecessary. In contrast, for purification and evaluation of specificity of the antigen, the criterion is the quantity of antigen that reacts with a constant amount of antibody, and experience with most immunological techniques is more limited in this area. Lipids present a special problem because of their limited solubility in aqueous medium, and this problem is magnified because, as will be indicated, lipid antigens may not be active except in the presence of other ("auxiliary") lipids. On the basis of experience with a number of lipid hapten systems—cytolipin H, rat lymphosarcoma hapten, Forssman hapten from horse kidney—the method of complement-fixation appears to be superior to others for following the course of lipid hapten purification. Using rabbit antiserum as the analytical reagent, complement-fixation is a general method for measuring the activity of lipid haptens with sufficient sensitivity to provide consistent results in most instances.[2]

One of the main drawbacks to the convenient application of this method has been overcome with the development of techniques for integrating complement-fixation data into a line curve (isofixation curve) that readily permits relations to be established between quantities of antiserum and antigen that react throughout the zones of antibody and antigen excess, and thus permits systematic comparison of antigens (6). As has been pointed out, satisfactory measurement may require adjustment of the sensitivity to conform to the properties of both antiserum and antigen.

[2] The test of consistency is the comparison of high concentrations of weakly active fractions with lower concentrations of more active fractions.

This sensitivity is determined by the quantity of complement used in the test. The highest level of sensitivity (2 units of complement) is inadvisable because it subjects the system to excessive nonspecific interference. On the other hand, use of too much complement may lower the sensitivity to the point where available antisera are not sufficiently strong to produce a desirable intensity of fixation. It would be difficult to eliminate the abstract quality of these statements without giving concrete examples, and, indeed, immunologists who may not have had extensive experience in antigen purification have unfortunately failed to appreciate the significance of isofixation curves and the need for flexibility in the adjustment of sensitivity level (7).

In attempting to fractionate crude lipids and examine individual fractions for specific activity, two observations that give this kind of problem an individual flavor come to the fore. In a larger sense, these observations, which appear to be general, may have been the chief obstacles to the systematic development of this field. These observations are first, that individual fractions may be intensely anticomplementary (and thus not subject to immunological evaluation), whereas the original crude lipid mixture, in sharp contrast, shows none of this property; and second, that as fractionation proceeds, the total activity recovered markedly decreases, and the intensity of fixation is also diminished. Pangborn's enormous achievement in isolating the first pure lipid hapten, cardiolipin, in the face of these difficulties (8, 9) was of inestimable value in providing a concrete example of how they might be overcome. Pure cardiolipin, a phosphatidic acid, was anticomplementary, and this undesirable property could be eliminated by admixture with lecithin. The combination of lecithin and cardiolipin was not particularly reactive with reagin (human antibody appearing during the early phase of syphilitic infection) but the sensitivity could be increased to almost any desired degree by adding cholesterol. It must be pointed out that the addition of lecithin or cholesterol to "strengthen" haptenic activity of lipid extracts had long been one of the "tricks of the trade" of those engaged in studies of this nature. Their results were, however, strictly empirical since such auxiliary lipids were added to mixtures of undefined content, and in some cases addition of lecithin to different lipid mixtures caused decreased reactivity of some and increased reactivity of others with the same antiserum (10). The demonstration that auxiliary lipids were required with a pure hapten was the first step in appreciating this phenomenon to be a characteristic feature of the reactivity of lipid haptens in complement-fixation. The anticomplementary activity of cardiolipin may have a relation to its acidic properties. Pure cytolipin H, on the other hand, is a neutral molecule and is not anticomplementary; but it too is unreactive in complement-fixation unless combined with auxiliary lipids, a mixture of lecithin and cholesterol being most effective (2). Lecithin and cholestrol may not be unique in enhancing activity of all lipid haptens. Landsteiner and Levene (11) used brain sphingomyelin to increase the serological activity of purified preparations of Forssman hapten. A similar effect was obtained by Papirmeister and Mallette (12) by the addition of phrenosine to a sheep red cell hapten. However, neither pure sphingomyelin nor phrenosine enhanced cytolipin H activity.[3] It will be of considerable interest to determine the extent to which auxiliary functions differ with different pure lipid haptens. The antiserum plays a role here also, and it has been observed in some experiments that changing the relative proportions of auxiliary lipids produces different effects on reactions of the same (pure) hapten with different antisera.[1]

The requirement for auxiliary lipids is most pronounced for *formation of immune complexes that will fix complement*, and, indeed, a quantity of auxiliary lipid that is 50 to 100 times the quantity of pure lipid hapten (cytolipin H)) may be required for maximum effect (2). The role of lipid interactions is not, however, restricted to this phase of immunological activity. Some studies indicate that the *binding of pure lipid hapten with antibody* also requires the presence of other lipids (2, 13). A definitive study of this phenomenon has not yet been made; however, there are reports indicating that lecithin alone is effective with both cardiolipin (13) and cytolipin H;[3] with the latter substance, cholesterol alone is ineffective[3] and does not contribute to the effectiveness of lecithin. The quantity of auxiliary lipid producing a maximum effect in hapten-antibody *binding* may be very much less than that required for *complement-fixation*, and this may in part account for puzzling observations with partially purified lipid fractions which showed activity by absorption methods but not by complement-fixation (14).

Preliminary experiments with cytolipin H[3] suggest that lipid-lipid interactions may also be important in a third aspect of immunological activity, namely, *elicitation of antibody response*. This phenomenon, which is really far too complex to be considered in the same category with the others, has been studied with lipid

[3] M. M. Rapport and L. Graf, unpublished observations.

extracts in the past by the method of "combination immunization," namely, the use of foreign protein (swine serum) as a carrier. The findings in such experiments have been summarized in the last comprehensive review devoted exclusively to lipid haptens (15). However, using limited quantities of pure cytolipin H, it was observed that this method did not evoke antibody response, even when cytolipin H was combined with pure auxiliary lipids.[1] In contrast, antibody to cytolipin H was obtained when a mixture of cytolipin H and crude tissue lipids (and swine serum) was injected.[1] It is not difficult to understand why this phase of the immunological study of lipid substances has always been controversial. With the exception of synthetic lipids and cholesterol, studies in the past have usually been conducted with impure lipids, and evaluation of the final results has included the combined effects of several substances in the three distinct phases of activity mentioned above. We are not yet in a position to understand the bizarre observation of Levene *et al.* (16) that an antiserum to lecithin gave preferential reactions with cholesterol; when a sufficient array of natural haptens is available for study, such strange results may be found to appear so infrequently that they will not require emphasis. It is perhaps relevant here to recall that injection of tissue particulates, in which phosphatides and cholesterol are major lipid constituents and lipid haptens are present in only minor amounts, does not lead to the production of antibody that reacts specifically either with phosphatides or cholesterol.

CHROMATOGRAPHIC PURIFICATION OF LIPID HAPTENS

The technological advance represented by column chromatography on silicic acid for the separation of complex lipids is the single development offering the most promise for revitalizing this area of immunological exploration. With the exception of Pangborn's brilliant effort in the 1940's, this field of activity has been strangely silent since its weary protagonists abandoned the struggle almost a quarter of a century ago. With characteristic insight, Landsteiner had already perceived the new direction in which to move (3*c*). It is a matter of record that the application of this new tool to an old field was begun even in the process of shaping the tool (17). A study of the resolution of phospholipids on silicic acid, started in 1953 in an effort to separate various lipids with thromboplastic activity, was enlarged, later that year, to include the separation of the lipid hapten of rat lymphosarcoma. To date, the major achievement resulting from the use of this new method is the isolation of cytolipin H from human tissues. However, within the past year, studies of other systems have provided strong indications that a systematic method of general applicability may be at hand, whose sole limitations will be the concentration of a given hapten, the number of chemically similar haptens present in a particular source, and the structural complexity of the molecules themselves. Whether a direct approach to purification and characterization of lipid haptens is attainable depends in large measure on the degree to which generalization of hapten structure is valid. This subject will be discussed later. For the moment, let us consider a typical fractionation experiment in which crude lipids, whose water-soluble contaminants have been removed, are subjected to fractionation on silicic acid by gradient elution, the individual fractions then being assayed with antiserum by complement-fixation. Several experiments of this type have been described (18, 19). What will generally be observed is that immunological activity is associated with two groups of fractions, namely, those eluted rapidly with the neutral lipids, and those eluted more slowly with the amino-phosphatides. The first group will be found to be strongly anticomplementary, whereas the second group will not. If, before making the aqueous suspension of these fractions, some lecithin (in an organic solvent) is added to each, the fractions of the first group will usually lose their activity, whereas activity will be retained by those of the second group. This procedure is not entirely satisfactory for two reasons. It does not fractionate the lipids in any systematic way, and the quantity of lecithin added bears no constant relation to the quantity of material in the test fraction. Both of these undesirable features may be readily eliminated. In the first place, by using a discontinuous gradient elution scheme, a smaller number of lipid fractions may be collected; these will show the properties of the known lipids constituting the bulk of the material under study. The final fraction, eluted with methanol, will probably be inactive and consist of choline-containing phosphatides. This fraction may be used as the source of lecithin. Now if all fractions are made up to the same volume, then by combining equal volumes of any given fraction with the last fraction (choline-containing phosphatides), the amount of lecithin added will be in approximately the same proportion to the active lipid as it is in the original crude lipid mixture. We have thus available a systematic method of examining the total lipid extract of an organ or tissue for its hapten content with respect to the analytical reagent,

an antiorgan serum. The number of active substances found will depend on three factors: the specificity of the antiserum, and the number and relative concentrations of haptens in the original lipid extract. This procedure has recently led to the confirmation of the presence of a novel lipid hapten in human tissues,[4] the existence of which was indicated (20) in an earlier study of antihuman tumor serum specificity.

STRUCTURE OF LIPID HAPTENS

It seems reasonable to predict that the adsorption affinity of lipids for silicic acid will prove to be a more substantial criterion of lipid structure than solubility properties, which are so frequently employed even today. When it was found that a rat lymphosarcoma hapten was eluted from silicic acid just after the main aminophosphatide fraction (17), it was assumed that this hapten would prove to be a phosphatide (18). This thinking was influenced considerably by the fact that cardiolipin, the only pure animal lipid hapten known, was a phosphatide. Cytolipin H activity was eluted just before the main amino-phosphatide fraction (19), a position consistent with that expected for a phosphatidic acid like cardiolipin. The active fraction as eluted from the column contained considerable phosphorus, but the pure substance was subsequently found to be free of phosphorus. This observation was influential in reorienting our thinking with regard to the structure of lipid haptens, despite the suggestive evidence based on adsorption affinity for silicic acid.

Let us now consider the structures of the two pure haptens so far obtained from animal tissues. On the basis of analyses of the sodium salt of cardiolipin for carbon, phosphorus, fatty acids, glycerol, and sodium and the identification of a polyglycerol ester of phosphoric acid among the hydrolytic products, Pangborn (21) suggested the structure of cardiolipin to be glyceryl-phosphoryl-glyceryl-phosphoryl-glyceryl-phosphoryl-glycerol, in which each of the six available hydroxyl groups on the four glyceryl residues was esterified with an unsaturated fatty acid, five with linoleic acid, and one with oleic acid. Very recent evidence based on oxidative degradation of deacylated polyglycerophosphate (22) and paper chromatographic methods (23) suggests the correct structure of the molecule to be simpler, namely, glyceryl-phosphoryl-glyceryl-phosphoryl-glycerol, in which each of the four available hydroxyl groups on the two terminal glyceryl residues is esterified with fatty acid, and a single hydroxyl group on the central glyceryl residue is free (Fig. 1). Problems in the preparation of this material and its use in serodiagnosis of syphilis have been reviewed with great clarity by Pangborn (25, 26, 27). She has emphasized the effects of lipid-lipid interactions and noted that the quantity of cardiolipin in the optimally reacting mixture is small in proportion to the quantity of auxiliary lipid, and further, that specificity as well as sensitivity depend on the proportions of the three components (cardiolipin, lecithin, cholesterol) (25). Pangborn utilized data obtained by both complement-fixation and flocculation methods for estimating the reaction of cardiolipin with human antibody. Studies in recent years have extended such observations to rabbit antibody and the quantitative precipitation method (13). The value of lecithin apart from that of overcoming anticomplementary activity was noted (13). It has also been found (28) that antibody reacting with cardiolipin will react (fix complement) with relatively large amounts of lecithin, although substantial differences in reactivity were recorded among three lecithins (two synthetic, one naturally occurring) tested with the same antiserum. It was not suggested that this represents a true cross reaction. Such observations emphasize another limitation in the study of lipid specificity, namely, that, compared with water-soluble substances, specific reactions must be judged over a narrower range of concentrations. This is so because we are not yet able to determine what quantities of various lipids react nonspecifically with complement components, reactions that may occur because one of these components is a lipoprotein.

Little information is as yet available on the extent to which structural analogues of the cardiolipin molecule react with anticardiolipin. On the basis of the revised (diphosphatidyl glycerol) structure, the glyceryl phosphatide models prepared by Baer and Buchnea (29) assume increasing relevance and their activity is not unexpected. It may be pointed out that the new cardiolipin structure, in contrast to the former one, offers a large segment of polar residues (phosphate-glycerol-phosphate) unhindered by hydrocarbon chains, and this feature may be of considerable aid in eventually accounting for the specificity of cardiolipin reactions.

After it had been isolated in a pure state, cytolipin H was found to be composed of four molecular residues: fatty acid, a long-chain base, glucose, and galactose in equimolar proportions (1, 2). Since then the fatty acid residues have been established by paper chromatography and gas-liquid partition chromatog-

[4] L. Graf, M. M. Rapport, and R. B. Brandt, unpublished studies.

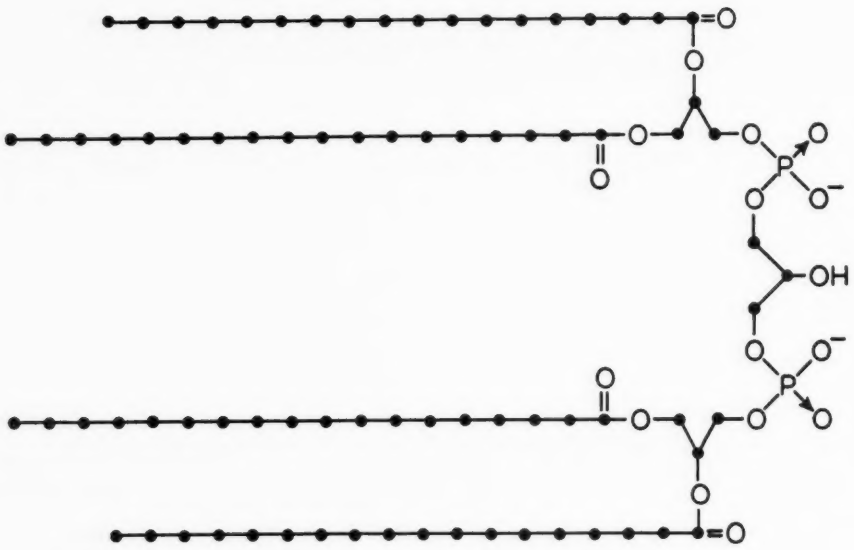

FIG. 1. The structure of cardiolipin (22, 23, 24).

raphy to be principally a combination of lignoceric, behenic, and palmitic acids (30); the long-chain base has been found to be sphingosine (30) by the decisive method introduced by Sweeley and Moscatelli (31); and the carbohydrate configuration has been established to be that of the disaccharide lactose using the sensitive immunochemical method of hapten inhibition (32). Structurally, cytolipin H is a lactoside of ceramide (Fig. 2), and it is therefore similar to cerebrosides in having a ceramide aglycon, but differs in being a glycoside of a disaccharide rather than a monosaccharide. This type of structure provides a simple solution to the problem of how a high degree of immunological specificity can be associated with lipids, which are relatively small molecules whose pattern normally permits only a limited number of structural variations. The combination of lipid residues with oligosaccharides permits a large number of possible variations to be incorporated into a class of small molecules which have the capacity to achieve high particle weights in an aqueous environment without the formation of covalent linkages.

Before considering what special assets accrue to this unique structural design and what advantages may be taken of this, let us first ascertain whether either of the two pure hapten models, phosphatide or glycosphingolipid, offers indications for the generalization of chemical structures with haptenic properties.

OTHER PROBABLE SPHINGOLIPID HAPTENS OF ANIMAL TISSUES

In their last communication on the "heterogenetic hapten" of horse kidney, Landsteiner and Levene (33) reported that the purest material they had obtained was free of phosphorus and that carbohydrate was present among the hydrolysis products. Similar results were recorded by Brunius (34). A lipid hapten from bovine brain was reported by Schwab (35) to be a "protagon" fraction, i.e., an ether-insoluble fraction in

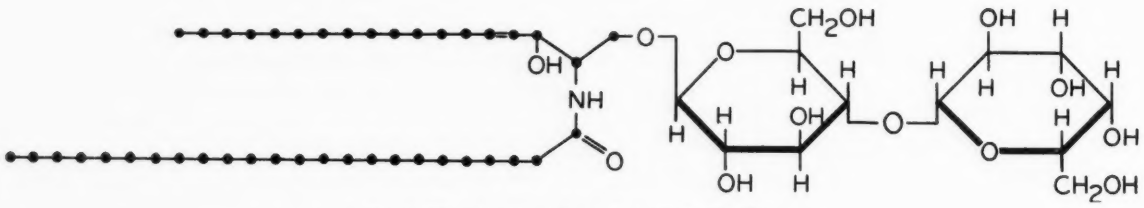

FIG. 2. The structure of cytolipin H (2, 30, 32).

which sphingolipids (usually sphingomyelin and cerebroside) are concentrated. The haptenic activity of a lipid isolated from bovine spleen, lung, and liver, and thought to be "polydiaminophosphatide" (36), a chemical structure that is no longer valid (37), was clearly associated with a sphingolipid fraction. Much of the effort directed toward isolation of blood group substances from erythrocytes of both horse and man has yielded glycosphingolipid fractions (38, 39), and most recently, a sheep erythrocyte lipid with Forssman hapten activity was found to belong to this group of compounds, since it contained both carbohydrate and lipid base residues (40). With the exception of the last substance, there is little to be gained by detailed discussion of these results since pure materials were not obtained. The sheep erythrocyte hapten, however, was reported to be pure and will be discussed below.

No comparable list implicating phosphatides as lipid haptens of animal cells can be assembled at present. Admittedly, the list of sphingolipids is weighted, since careful reading of the papers indicates some of these fractions to be water-soluble; and, according to our operational definition, such substances might not be classified as lipids since they would be expected to appear in the methanol-water layer rather than the chloroform layer after washing the chloroform-methanol extract. This would be particularly true of erythrocyte glycosphingolipids as the number of monosaccharide residues increased. In any case, it is apparent that there is a high probability that glycosphingolipids are a general model for animal cell haptens. If one attaches carbohydrate residues to lipid residues, can the resultant molecule still be classified as lipid? At what point does glycolipid become lipopolysaccharide? This question cannot be easily answered. Aside from indicating the procedures that one employs to isolate, characterize, and study the chemical and physical properties of a substance, the question has little meaning. In the case of cytolipin H, it is clear that chemically the substance behaves as a lipid, whereas immunologically it reacts principally as a carbohydrate. What is most suggestive about this design of at least some cellular haptens as glycosphingolipids is related to the finding that sphingolipids such as sphingomyelin and cerebroside are almost certainly myelin components, and therefore presumably components of membranes. In ceramide we have two lipid residues by means of which an oligosaccharide label can be cemented into the lipophilic portion of a cell membrane. This label, which is now part of a high particle weight structure by virtue of the mass of the cell, may then exercise its influence in a variety of reactions, which are most familiar to the immunologist because of the existence of antibodies which produce agglutination, complement-fixation, and cytotoxicity. Much of the serological work on individual cell labels has been restricted to erythrocytes because of their obvious technical advantages (discrete cells, self-contained pigment). These cells have proved to be an unsatisfactory source of material for most chemical studies for reasons which are both known (difficulty in removing hemoglobin) and suspected (low concentration of individual hapten; presence of many haptens). Studies by several groups of investigators (41 to 45) suggest the presence in erythrocytes of many substances that may have haptenic function. It is to be hoped that cells grown in culture will offer a far simpler source of material for chemical study in much the same way as have tumor tissues.

If most lipid haptens should be glycosphingolipids, there would be a simplification in their isolation and identification. As a result of very recent studies (17)[4,5] a number of lipid haptens (from rat lymphosarcoma, horse kidney, human colon carcinoma) were found to be eluted from silicic acid together with aminophosphatides. Because of present analytical limitations, the further fractionation of phosphatides in this material would be exceedingly difficult. However, the separation of a sphingolipid from the phosphatides is relatively straightforward, and when this was done with Forssman hapten from horse kidney,[5] the active fraction was indeed found to be associated with carbohydrate-containing lipid. Purification can thus be based on carbohydrate content, and should specific monosaccharide residues be identified, an even more decisive criterion would be available. Furthermore, it is possible to gain some valuable information on the number of monosaccharide residues present in the molecule from the solvent required to elute the lipid from silicic acid, assuming no charged chemical groupings are present, such as sulfate, phosphate, or carboxyl (sialic acid). Discrete separations can be made between sphingolipids containing one and two monosaccharide residues, and between the latter and the main ninhydrin-positive phosphatide fraction (46).[6] The three haptens listed above, if they are glycosphingolipids, should contain only three, or possibly four, monosaccharide residues. This degree of molecular complexity is capable of exact solution with relatively small quantities of material. The main obstacles to be overcome are the poor criteria of purity and the shortage of techniques

[5] M. M. Rapport, L. Graf, and N. F. Alonzo, unpublished observations.

[6] M. M. Rapport and N. F. Alonzo, unpublished observations.

for purification, the latter resulting principally from the limited solubilities of these molecules. This view may be optimistic because it leans heavily on experience associated with the isolation of cytolipin H, whose structure, in retrospect, appears to be the simplest prototype. The properties of this substance do not differ appreciably from those of cerebrosides, so that a very considerable background of chemical experience is applicable to this hapten. The only serious loss in applying to cytolipin H methods useful for characterization of cerebrosides was the failure to gain useful information from infrared spectra. One of the intriguing aspects in the study of more complex lipid haptens is how chemical properties will be modified as additional monosaccharide residues are added. With the availability of synthetic methods (47) it may not be necessary to await isolation of natural products for complete answers, although, at the moment, the approach based on identification of natural products still offers an advantage in efficiency and considerably more from the standpoint of possible application.

Exact knowledge of the structure of lipid haptens, which is possible because of the combination of small size and basic simplicity of design, has an immediate consequence in indicating methods for producing specific antibody which circumvent the haptenic limitation. Antibody specific for a carbohydrate determinant can be prepared by immunization with protein chemically coupled to an oligosaccharide through an azophenyl linkage (48). Thus antisera prepared against bovine -γ- globulin-azophenyl lactose, or porcine -γ- globulin azophenyl lactose, that react with the lactose determinant as shown by precipitation with egg albumin-azophenyl lactose, also react with cytolipin H.[7] Studies are in progress which have as their objective to compare the avidity for cytolipin H of antibody directed against a lactose determinant and of antibody prepared against tissue fractions containing cytolipin H. If the avidity of the latter is found to be greater, as preliminary results suggest,[7] it would indicate that antibody formed against cytolipin H is complementary to part of the lipid structure (possibly the polar groupings of sphingosine) as well as the carbohydrate. With haptens containing three monosaccharide residues it might be expected that the proportional contribution of the lipid would be measurably less, and that a higher degree of specific reaction with antibody would be observed. Increasing the number of carbohydrate residues should also affect antigenic strength, as expressed in the capacity of a mixture of haptens associated with protein (either in tissue fractions or in "combination immunization" techniques) to elicit antibody reacting with individual components. The phenomenon of antigenic competition among haptens, sometimes shrouded with the nebulous concept of "availability," is indicated in experiments in which cytolipin H was found to be a poor competitor in the presence of a rat tissue hapten of greater chemical complexity.[1]

[7] L. Graf, J. Yariv, and M. M. Rapport, unpublished observations.

SPECIFICITY OF LIPID HAPTENS

The specificity to be considered here is one of distribution: Are these relatively low molecular weight lipid haptens found only in specific cells, and if so, are they present in corresponding cells of different animal species? If the molecular residues composing these haptens are part of the ordinary pool of structural components and the hapten pattern is so simple, how many variations occur and how often is the same pattern used? The answer to these questions is of paramount importance for our understanding of the biological significance of lipid haptens or appreciation of how they may be used for some practical purpose. Answers are not readily deduced from available evidence because of inherent defects in the methods employed to obtain the data. For example, extensive surveys have so far been limited to Forssman haptens and blood group haptens (49, 50, 51) (cardiolipin is distributed almost ubiquitously). Neither of these two types of hapten activity represents a discrete chemical entity. Both human A and B group specific substances are present in red cells and tissues as either alcohol-soluble substances (presumably lipid haptens) or as water-soluble peptide-polysaccharide combinations (50, 51). Forssman haptens are defined as substances which react with antibody to sheep erythrocytes; if the antigen mosaic of these cells is complex, then many different molecules, either identical with one of the erythrocyte antigens or capable of cross-reacting with antibody to them, will be included in any measurement of "Forssman hapten." Furthermore, antisheep erythrocyte sera may vary qualitatively and quantitatively in antibody content. It is small wonder, therefore, that the distribution of Forssman hapten has been found too broad to be instructive. Nevertheless, it is clear that studies of the Forssman system established that lipid haptens have the property of crossing species lines, and, as a consequence, the terms "heterogenetic" and "heterophile" were introduced. Although these terms are sometimes used synonymously with "Forssman," they have a broader meaning since they

are applicable whenever haptenic activity in one species is observed with an antibody system "homologous" to a different one. However, this terminology is so ambiguous that organ specificity (in the sense of reactions common to a tissue such as brain or heart, regardless of the animal of origin) is not referred to in the literature as "heterophile" (52). Such terms reflect the inability of serological methods per se to supply definitive answers. Chemical methods may eliminate much of the confusion, but a combination of chemical and serological analyses will doubtlessly be required to secure stable evidence. It is worth mentioning that the distribution of individual lipid haptens may not be as broad as studies of the Forssman model indicate. The rat lymphosarcoma hapten system, for example, showed no cross reactions with lipid extracts from tissues of five heterologous mammalian species (53).

Limitations of serological methods are attributable to imperfect specificity of antisera and unreliability of quantification. Their strength lies in sensitivity. Chemical methods, on the other hand, offer reliable quantification but suffer from lack of sensitivity, and also from lack of specificity although the range of imperfection is narrow. The two methods can complement each other nicely if the limitations are kept in mind. A combined serological-chemical approach to analysis of lipid hapten distribution has been started with the cytolipin H system using one of the more specific antihuman tumor sera.[3]

Before discussing some of the early results, let us first look at an interesting facet of chemical investigation that is a direct consequence of isolation and structural identification of cytolipin H. A molecule with similar composition had been isolated from bovine spleen by Klenk and Rennkamp in 1942 (54) as a by-product of the study of gangliosides, and it was quickly found[1] that lipid extracts of this organ did indeed react with antisera that gave reactions with cytolipin H. Rigorous confirmation by chemical and serological comparison of the substances was required, and therefore several pure preparations of an active glycolipid were isolated from bovine spleen. These preparations and cytolipin H gave very similar values on chemical analysis, and small differences in rotation and unsaturation that were found could be attributed to variations in the lipid residues (55). Although more than 90% of the lipid base was sphingosine and the fatty acids were predominantly lignoceric (C_{24}), behenic (C_{22}), and palmitic (C_{16}) acids, the proportions varied from one preparation to the next, as did the other fatty acid residues. Serologically the material from bovine spleen was indistinguishable from that isolated from human tumor (55). An earlier report became especially interesting now, namely, that a pure sheep erythrocyte hapten had been found to contain fatty acid, a lipid base, galactose, and galactosamine (40), and it therefore seemed probable that an increasingly large number of different glycosphingolipids with two monosaccharide residues might be found as isolation and chemical identification of lipid haptens progressed. For this reason the generic term "cytoside" was introduced (56) to simplify discussion of these different molecules and their immunological individuality. The term cytoside to describe a glycosphingolipid with two monosaccharide residues is similar to the term cerebroside, which is commonly used to describe a glycosphingolipid with one monosaccharide residue. Introduction of such a term has additional validity on the basis of chemical classification based on adsorption affinity for silicic acid. As a result of studies of an appreciable number of human tissues and a limited number of animal tissues, it is apparent that cytosides are much more rarely encountered than had been surmised.[3] Furthermore, the description of the sheep erythrocyte hapten as a cytoside may be questioned for the following reason. Cytolipin H and bovine spleen cytolipin are similar to cerebrosides in their solubility characteristics. In particular, they are completely insoluble in water. The sheep erythrocyte hapten, in contrast, is reported to be water-soluble (40), and it is difficult to reconcile the small differences in chemical composition with such a profound difference in physical properties unless the chemical structures differ greatly. Using a combination of chemical and serological analyses, both sheep and dog spleens were found to contain small quantities of cytoside having the serological reactivity of cytolipin H.[3] However, rodent (rabbit, rat, mouse), cat, hog, and horse spleens were found to be devoid of cytolipin activity and to show no indication of the presence of other cytosides.[3] The findings suggest a slight revision in terminology. The H in cytolipin H, originally introduced to indicate "human," should therefore be considered to represent "heterophile" or "heterogenetic."

Among human tissues, examination of a limited number of specimens showed cytolipin H to be absent from brain, heart, kidney, and a number of tumors.[3] This hapten was found also to be one of the glycosphingolipids of pooled horse erythrocytes but not of bovine or human erythrocytes (43). These results suggest that lipid haptens are associated with particular cells, that a given structural pattern may be repeated in different species, and that distribution may be much more restricted than earlier studies of Forssman

hapten led us to believe. Obviously much more work will be required before we can formulate valid generalizations.

LIPID HAPTENS OF HUMAN TISSUES

How many different lipid haptens are found in cells of a single species? Extensive studies are available only for those of human origin. We can be reasonably certain of seven: cytolipin H, cardiolipin, the hapten in tumors of the gastrointestinal tract previously referred to, a Forssman hapten, the alcohol soluble group A and group B substances of erythrocytes, vascular intima, islets of Langerhans, gastric parietal cells, and lung (50, 51), and the Rh hapten (57, 58). Five of these must still be isolated, characterized, and identified. There are, in addition, at least two other lipid haptens that appear to be organ specific (in the sense that similar tissue from a different species of animal contains significant quantities), namely, one from myocardial sarcoplasm that can be distinguished from cardiolipin (59, 60), and one from brain (61, 62). It is probable that these nine substances will be serologically unrelated, but studies directed toward answering this question have not yet been reported. Other potential haptens exist wherever carbohydrate residues are found bound to lipids, such as the cytosidelike fraction found in low concentration in human kidney (that does not show cytolipin H activity).[3] It is not, therefore, an unwarranted assumption that many individual cell types may have distinguishing hapten labels in their membranes by means of which serological identification can eventually be made. The number of lipid haptens may be increased greatly by including those not present in man, some of which are indicated in an earlier review of heterophile reactions (63). The fact that many of these substances are found in different animals offers a remarkable opportunity for overcoming the greatest obstacle in isolation work—finding an adequate source. This fortunate state of affairs actually led to the isolation and identification of the cytolipin molecule seventeen years before its haptenic function was established.

THE ROLE OF LIPID RESIDUES IN LIPID HAPTENS

As has already been indicated, the part played by the lipid residues in lipid haptens is poorly understood. From a theoretical standpoint, these residues may well serve as points of attachment of the haptens to organized segments of cellular structure such as membranes. There may therefore be no well-defined lipoprotein molecule of which they form a part and which was suggested by evidence based on complete antigenicity of cell fractions (64). Other, still preliminary, evidence implicates the participation of a portion of the lipid residue in the fragment of structure to which the antibody site is complementary.

From a practical standpoint, the presence of lipid residues in lipid haptens requires a distinctive approach in correlating structure with activity, and even for tracing activity during purification procedures. Lipids apparently display their specific reactions with proteins far more efficiently in concert with other ("auxiliary") lipids than alone. The effect of lipid-lipid interactions may be interpreted only in part on the basis of increased dispersion in an aqueous medium. The effect of cholesterol, a more hydrophobic substance than the hapten itself, and a common constituent of membranes, cannot be explained in this way. The requirement for auxiliary lipids is even seen in lipid-protein interactions involving lipids that disperse readily in water (12, 33, 34). Although only superficial information is available, there are indications that auxiliary lipids (lecithin, cholesterol, sphingomyelin, cerebroside), despite their ubiquitous distribution, may specifically activate different haptens. In part, the auxiliary lipid requirement for immunological activity is readily understandable on the basis of converting a monovalent molecule into a polyvalent aggregate, which can then react with antibody to form an immune complex with properties similar to those formed by water-soluble polyvalent antigens and antibodies. What is loosely thought of as "dispersion" may, in fact, be the formation of mixed micelles with optimally reactive molecular arrangements.

In studies with substances containing hydrophobic chains, it should also be appreciated that a considerable driving force favoring reaction may be associated with decreased water-solubility (65). It might thus be expected that the inhibition of lipid hapten-antibody reactions by water-soluble oligosaccharides would be less efficient than inhibition of polysaccharide-antibody reactions.

PSEUDO ANTIGENS AND OTHER LIPIDS

This review will close on a disagreeable note, namely, an attempt to separate true immunological reactions from studies which appear in some vague way to be relevant, but are not. Such studies provoke numerous questions on whether a relation exists among them because they all involve lipids associated with the

problem of cancer. Thus within a rather short period of time, in addition to cytolipin H, malignolipin (66), carcinolipin (67), a "crystalline antigenic phospholipid" (68), and "lipid antigens" useful in serodiagnosis (69, 70, 71) have been described. These appear to be unrelated. Cytolipin H has been discussed at length in this review. It is not "cancer specific," despite inadvertent misrepresentations to this effect (7). Malignolipin is presumably a phospholipid; although isolation of the pure substance was reported, adequate physical and chemical characterization was not presented. It has not been found to have antigenic or haptenic properties. Carcinolipin is the name assigned to a lipid of undefined structure that was detected originally in egg yolk, because of its enhancing effect on protein biosynthesis. It is present in rat tissues and is elevated in tissues of tumor-bearing animals. No immunological properties have been associated with this material. The so-called "antigen" of the Penn test (69), and a similar phenomenon described by Eisenstaedt (70, 71), is not an antigen. This terminology is used because the technique of the test bears a resemblance to serofloccculation methods that have been used with some lipid haptens. While the physicochemical basis of the reaction is not well understood, it appears to involve an effect of serum proteins in disturbing a metastable lipid sol. The protein composition of cancer sera may be such as will cause the sol to flocculate. Certain proteins appear to be more effective than others (72), and several lipids may be used to prepare the sol (73). The use of the term antigen here is indeed unfortunate. Finally a "crystalline antigenic phospholipid" is reported to have been isolated from human cancer tissue (68). This study does not appear well grounded and further consideration must await independent confirmation of the findings. It is essential to keep these different studies well separated in order to avoid prejudging the merits of any one on the basis of the success or failure of the others. Aside from the value that new substances offer in terms of potential utility, much more substantial gain may attend attempts to place them in proper perspective by chemical and physical characterization. It is essential to establish the nature of the reactions in which these lipids participate, if we are to expect any rational exploitation of their specific properties.

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A new method for the analysis of component mono-, di-, and triglycerides*

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SUMMARY

A new micromethod is described for the determination of component mono-, di-, and triglycerides. The basic procedure involves ozonization of the double bonds and catalytic reduction of the ozonides followed by separation and quantification of the glyceryl residues by thin-layer chromatography. The potentialities of the method are demonstrated by the analysis of soybean oil and lard as well as standard mixtures of synthetic mono-, di-, and triglycerides. Procedures for the analysis of the four monoglyceride types, six of the seven possible diglyceride types, and four of the six possible triglyceride types are demonstrated.

Present methods for the determination of component triglycerides involve separation of the fat into fractions of simple composition. The analysis of the constituent fatty acids of these fractions forms the basis for calculating component triglyceride composition. Hilditch and Lea (1) and Kartha (2, 3) carried out the fractionation after oxidation with permanganate. The separation also has been accomplished directly by fractional crystallization (4 to 7) and countercurrent distribution methods (8 to 11). Lipase hydrolysis has been used to obtain information on glyceride structure (12, 13), and may be used in conjunction with the latter methods to provide more detailed information on glyceride composition.

At a recent meeting of the American Oil Chemists' Society two other methods for the determination of glyceride structure were reported. One employed thermal gradient crystallization (14), and the other, an oxidation procedure in which the oxidized fat was separated into two fractions and analyzed by lipase hydrolysis and gas-liquid chromatography (15).

Methods for the determination of mono- and diglycerides are many and varied. However, except for the determination of 1- and 2-monoglycerides (16, 17, 18), these methods do not normally distinguish between the different types of these compounds.

Described here is a general procedure which may be applied on a microscale to the analyses of the individual types of mono-, di-, and triglycerides. The method involves the quantitative fission of the double bonds to yield aldehydes, followed by analysis of the glyceryl residues by the elegant technique of thin-layer chromatography applied to lipids by Mangold and Malins (19) and Mangold (20), and will be referred to in the text of this paper as the ozonization-reduction-thin-layer chromatographic method of analysis.

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MATERIALS

The following reference compounds were prepared in highly purified form: 1-monoolein (m.p. 34.9°-35.2°), 1,3-diolein (m.p. 25.0°-25.8°), triolein (I.V. 85.7; Sap. Val. 189.9), 1-monopalmitin (m.p. 76.5°-77°), 1,3-dipalmitin (m.p. 69.8°-70.2°), tripalmitin (m.p. 64.9°-65.1°; Sap. Val. 208.8), glyceryl-2-oleate-1,3-dipalmitate (I.V. 30.2; m.p. 34.5°-34.8°), and glyceryl-1-palmitate-2,3-dioleate (I.V. 58.6; m.p. 19.8°-20.0°). In addition to these, 1,3-distearin (m.p. 79.5°-79.9) and 1,2-distearin (m.p. 69.5°-70.0) were obtained from A. de Freitas, of the Department of Biochemistry, University of Minnesota, and 2-monopalmitin was obtained from Dr. F. H. Mattson, of the Procter and Gamble Company, Cincinnati, Ohio. The distearins contained only traces of impurities. The sample of 2-monopalmitin contained 9.9% of the 1-isomer as an impurity, as determined by periodate analysis (21).

The 1-monoglycerides were synthesized by the method of Fischer (22). The crude products were recrystallized several times and then chromatographed

on a silicic-acid column (17) until they were homogeneous as determined by thin-layer chromatography (19, 20). Periodate analysis (21) indicated they were pure 1-monoglycerides.

The 1,3-diglycerides were prepared by the trityl synthesis (23, 24) and purified in a similar manner.

A mixed unsaturated-saturated diglyceride also was prepared by reacting oleyl chloride (prepared by reacting oleic acid with oxalyl chloride, and purified by distillation) with 1-monopalmitin by the Hartman procedure (25). Impurities were removed by silicic-acid column chromatography (17). This preparation (m.p. 37.5°-38.5°) consisted of one major component (84.4%) and one minor component (15.6%) by thin-layer chromatographic analysis. Presumably the major component was glyceryl-1-palmitate-3-oleate and the minor component glyceryl-1-palmitate-2-oleate (25).

Tripalmitin and triolein were prepared by transesterification of the corresponding pure methyl esters with triacetin, using sodium methoxide as a catalyst (26). Most of the excess of methyl ester and monoglyceride impurities was removed from the crude triglyceride by several extractions of a Skellysolve F solution of the product with 90% ethanol. The bulk of the diglyceride impurity was removed by low temperature fractional crystallization. Traces of impurities still remaining were separated by silicic-acid column chromatography (17).

The preparation of glyceryl-2-oleate-1,3-dipalmitate and glyceryl-1-palmitate-2,3-dioleate was carried out by reaction of oleyl chloride with 1,3-dipalmitin and 1-monopalmitin, respectively. This reaction was carried out according to the procedure described by Hartman (25), except that the chloroform solution of the reactants was refluxed vigorously for 6 hours, during which the chloroform was removed gradually by distillation. The crude products were purified by silicic-acid column chromatography (17) until they were homogeneous by thin-layer chromatography.

Alkali-refined soybean oil and a commercial sample of prime steam lard were obtained from Archer-Daniels-Midland Co., Minneapolis, and Geo. A. Hormel & Co., Austin, Minnesota, respectively. The minor constituents of these fats were removed by adsorption on a column of silicic acid.

GENERAL PROCEDURE

A small amount of sample is dissolved in about 30 ml of methylene chloride and ozonized at −60° to −70°, with a mixture of about 3% ozone in oxygen generated in a laboratory ozonizer similar to that described by Bonner (27). The mixture of oxygen and ozone is bubbled through the solution at about 100 ml per minute. Under these conditions the ozonization is complete in about 5 minutes. The reaction is stopped when the solution turns faintly blue, and pure nitrogen is bubbled through the solution to remove all the dissolved oxygen and ozone. Following this, hydrogen is bubbled through the solution at room temperature and maintained at a slight positive pressure by using a small bore outlet tube. About 50 mg of Lindlar catalyst (28) is added to the reaction mixture; stirring is effected by means of a magnetic stirrer.

After allowing about 30 minutes to complete the reduction of the ozonides, the catalyst is removed by filtration through a sintered glass funnel, and washed 2 or 3 times with 5 ml amounts of methylene chloride, which are added to the filtrate. With very small samples the volume of the filtrate is reduced to about 0.5 ml by evaporation under reduced pressure at room temperature, otherwise the solution may be analyzed directly.

A volume of the solution containing about 50 μg of glyceryl residues is spotted with a microsyringe on the base of one or more glass plates (2″ × 8″) having a thin layer of silica gel G prepared according to Stahl (29, 30). The plates are placed in a small glass jar containing the appropriate solvent system, and developed in ascending manner. Usually a developing time of only 10 to 20 minutes is sufficient to provide a separation of the components. After the plates are developed they are dried at room temperature, sprayed with 50% aqueous sulfuric acid, and heated on a hot plate to locate the position of the spots (31).

The spots were measured with a densitometer (Photovolt Corporation, 52-C and 521A) with a stage attached to it for semiautomatic plotting of curves. The slit size of the densitometer was 1 × 5 mm and readings were taken at each millimeter of travel over the length of the plate. No filter was used. Usually there was only a slight difference in the background density from one end of the plate to the other; it varied linearly over the plate and had no effect on the accuracy of the measurements.

The areas under the densitometer curves were found to be directly proportional to the amount of sample for the saturated triglycerides and the glyceryl residues of the unsaturated triglycerides (Fig. 1). However, the area given by compounds of one type of structure may not be the same as that given by the same amount of compounds containing other structures, even though the carbon densities are essentially the same. This is demonstrated in Figure 1 from a comparison of the curves given by tripalmitin, triolein, and the glyceryl residue of triolein obtained by the

ozonolysis and reduction procedure. The significance of this observation is that standard curves must be prepared for the analysis of compounds that do not give spots of the same densitometrically-determined response. Since the standard curve given by the glyceryl residue obtained from triolein (after correction for carbon lost by fission of the double bonds) and tripalmitin were essentially identical, it was not necessary to prepare standard curves for the analysis of mixtures of triglycerides.

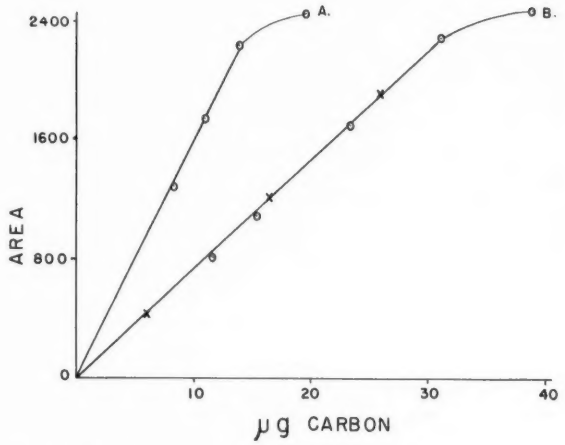
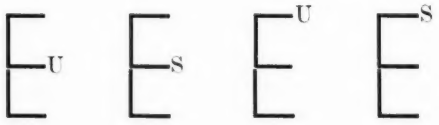


FIG. 1. Standard curve, thin-layer chromatographic analysis (*A*) triolein, (*B*) tripalmitin ○——○, and triolein x——x after ozonization-reduction (glyceryl residue corrected for fissioned carbon).

At present we have no explanation for the above observations but, under the conditions employed, unsaturation had a pronounced effect on the size and intensity of the spots developed on the plates.

Analysis of monoglycerides. There are four possible types of monoglycerides, viz.:



U and *S* in these models denote saturated and unsaturated fatty acid constituents. Alpha, i.e. (1-isomer) and beta, i.e. (2-isomer) monoglycerides can be determined by application of periodate analysis before and after perchloric acid isomerization (16). The relative proportions of the 1- and the 2-types also may be determined by applying silicic-acid column chromatographic analysis before and after oxidation with periodic acid (17), and by gas-liquid chromatography according to the procedure described by McInnes *et al.* (18).

Although compounds with small differences in polarity can be separated by thin-layer chromatography (19, 20), α- and β-monoglycerides cannot be separated from each other by direct application of this technique.

However, all four monoglyceride types may be determined by applying the analysis described here in conjunction with periodate oxidation. The general procedure is to conduct a periodic acid oxidation on an aliquot of the methylene chloride solution of the sample. The α-monoglycerides are oxidized to the corresponding glycol aldehyde esters by this reagent; the β-monoglycerides remain unchanged (17). This reaction may be carried out by any one of several procedures (17, 18, 32, 33). In this investigation the procedure for the analytical determination of α-monoglycerides was used (21). After the reaction was completed the solution was washed with distilled water, dried, and analyzed by thin-layer chromatography. The separation of 2-monopalmitin and glycol aldehyde palmitate obtained from 1-monopalmitin is shown in Figure 2 as an example of this analysis.

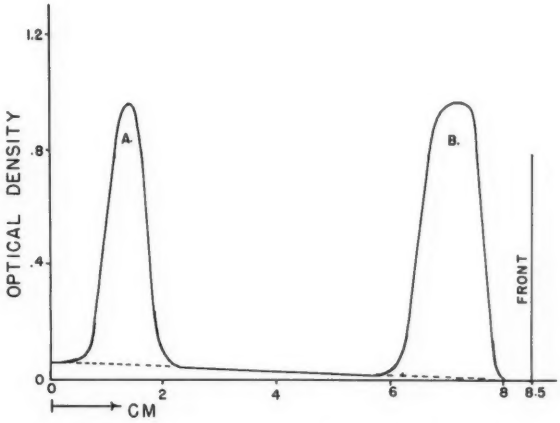


FIG. 2. Thin-layer chromatographic analysis of the products of the periodic acid oxidation of a mixture of 2-monopalmitin and 1-monopalmitin (solvent, 50% ethyl ether in Skellysolve F). *A*. 2-Monopalmitin. *B*. Glycol aldehyde palmitate.

The relative proportions of unsaturated and saturated β-monoglycerides may be determined by application of the ozonization-reduction-thin-layer chromatographic procedure to this solution. The determination of unsaturated and saturated monoglycerides by this method is demonstrated in Figure 3 by the analysis of a mixture of 1-monoolein and 1-monopal-

mitin. The analysis of unsaturated and saturated β-monoglycerides would give similar results.

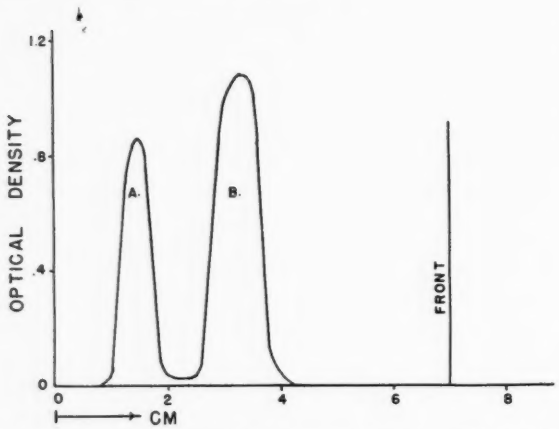
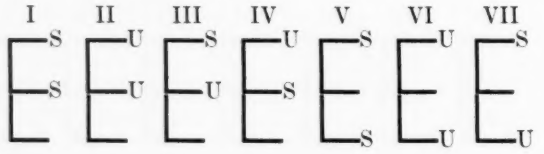


FIG. 3. Ozonization-reduction-thin-layer chromatographic analysis of 1-monoolein and 1-monopalmitin (solvent, 10% Skellysolve F in ethyl ether). *A*. Glyceryl residue of 1-monoolein. *B*. 1-Monopalmitin.

It may be noted that an analysis of the fatty acids of the glycol aldehyde esters and the 2-monoglyceride fractions recovered from a duplicate plate of the chromatographed periodic acid-oxidized fraction (Fig. 2) also would permit a complete characterization of each monoglyceride type.

Analysis of Diglycerides. These compounds may exist in seven different types, viz.:



S and *U* in these models also denote saturated and unsaturated constituents, respectively.

Application of the ozonization-reduction-thin-layer chromatographic method of analysis to these compounds gives a series of products that differ greatly in their polarity. The glyceryl residue of the unsaturated diglycerides (II and VI) are the most polar. The fully saturated diglycerides (I and V) remain unchanged and are the least polar; the mixed types (III, IV, and VII) have polarities intermediate between the fully-saturated types and the glyceryl residue of the fully unsaturated types.

The saturated diglyceride types (I and V) were analyzed by thin-layer chromatography with a solvent system consisting of 40% ethyl ether in Skellysolve F. With this solvent system the glyceryl residues from diglycerides containing unsaturated fatty acids remain at the base of the plates, and the simple aldehydes obtained from the fission of the double bonds migrate with the solvent front. The component saturated diglycerides (types I and V) may be analyzed with this solvent system, as demonstrated by the separation of 1,2-distearin from 1,3-distearin (Fig. 4).

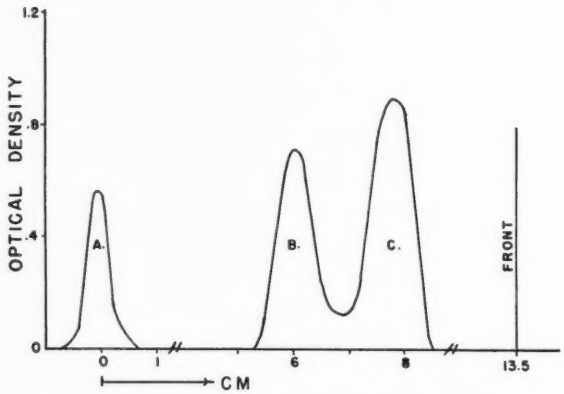


FIG. 4. Ozonization-reduction-thin-layer chromatographic analysis of 1,3-diolein, 1,2-distearin and 1,3-distearin (solvent, 40% ethyl ether in Skellysolve F). *A*. Glyceryl residue of 1,3-diolein. *B*. 1,2-distearin. *C*. 1,3-distearin.

The glyceryl residues from the unsaturated diglycerides may be analyzed by thin-layer chromatography with 35% Skellysolve F in ethyl ether. With this solvent system the saturated diglycerides and the aldehydes produced through fission of the double bonds migrate with the solvent front. Figure 5 shows the thin-layer chromatography of the glyceryl residues obtained from 1,3-diolein, glyceryl-1-palmitate-2-oleate, and glyceryl-1-palmitate-3-oleate representing the separation of diglyceride types VI, III, and VII, respectively.

Positional isomers like types III and VII have different polarities; the former, having a primary hydroxyl, is more polar than the latter, which has a secondary hydroxyl. These may be separated directly by thin-layer chromatography with the same solvent system used to separate 1,2-distearin from 1,3-distearin (Fig. 4). However, in order to avoid interference with the analysis of the fully saturated diglyceride types, the unsaturated linkages are converted to aldehydes by ozonization and reduction. The separation of the glyceryl residues of these types (III and VII) (Fig. 5) was not as good as the separation of 1,2- and 1,3-

distearin, but very likely this could be improved by the use of a slightly different solvent composition or by increasing the distance of the migration of the spots on the chromatographic plate, or both.

A sample of type II was not available for analysis but, in view of the difference in polarity between diglycerides containing a secondary and a primary hydroxyl group, no difficulty in separating type II from type VI was anticipated. Since type II would be more polar than type VI, it would have a lower R_f value and would migrate slower under the conditions of analysis used for the separations in Figure 5.

Types III and IV would have essentially the same polarity, regardless of whether or not they were ozonized and reduced, because each has one primary hydroxyl group and one unsaturated fatty acid constituent. Thus separation of these types from each other would not be possible. Nevertheless, it is evident that these types together can be separated from the other five types, giving, all told, the potentiality of analyzing six of the seven diglyceride types by the technique described.

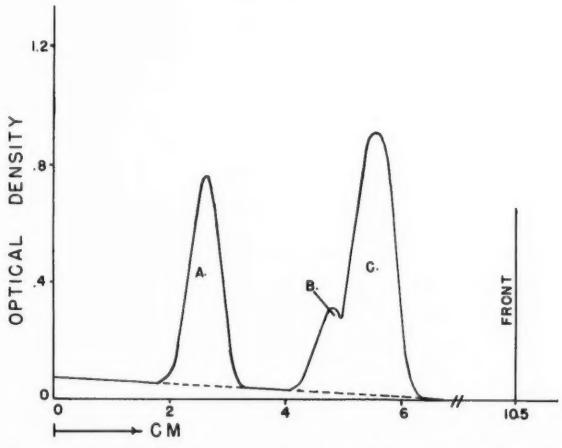


FIG. 5. Ozonization-reduction-thin-layer chromatographic analysis of 1,3-diolein, glyceryl-1-palmitate-2-oleate, glyceryl-1-palmitate-3-oleate (solvent, 35% Skellysolve F in ethyl ether). *A*. Glyceryl residue of 1,3-diolein. *B*. Glyceryl residue of glyceryl-1-palmitate-2-oleate. *C*. Glyceryl residue of glyceryl-1-palmitate-3-oleate.

Analysis of Triglycerides. Figures 6 and 7 show the results of the application of the ozonization-reduction-TLC method of analysis to a mixture of triglycerides of known composition consisting of tripalmitin (GS_3), glyceryl-1-palmitate-2,3-dioleate (GS_1U_2), glyceryl-2-oleate-1,3-dipalmitate (GS_2U_1), and triolein (GU_3). The glyceryl residues of the GU_3, GU_2S_1, and the GU_1S_2 were developed on one plate (Fig. 6) with a mixed solvent of 35% ethyl ether in Skellysolve F. The GS_3 and aldehydes fissioned from the constituent unsaturated fatty acids migrated with the solvent front with this solvent system. The GS_3 and the glyceryl residue of the GU_1S_2 were developed on another plate (Fig. 7) with 15% ethyl ether in Skellysolve F. The glyceryl residues of the GU_3 and the GU_2S_1 did not move from the base line with this solvent system.

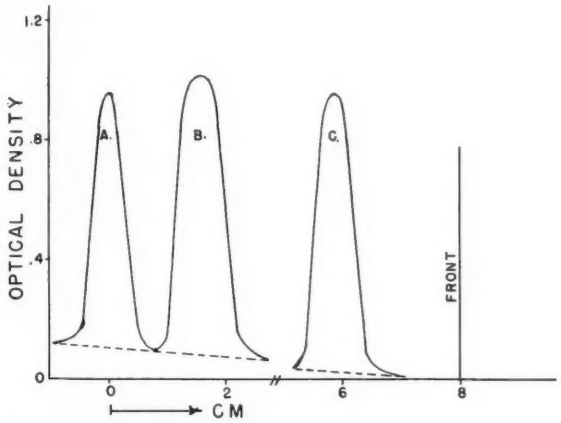


FIG. 6. Ozonization-reduction-thin-layer chromatographic analysis of trilolein, glyceryl-1-palmitate-2,3-dioleate, and glyceryl-1,3-dipalmitate-2-oleate (solvent 35% ethyl ether in Skellysolve F). *A*. Glyceryl residue of triolein. *B*. Glyceryl residue of glyceryl-1-palmitate-2,3-dioleate. *C*. Glyceryl residue of glyceryl-1,3-dipalmitate-2-oleate.

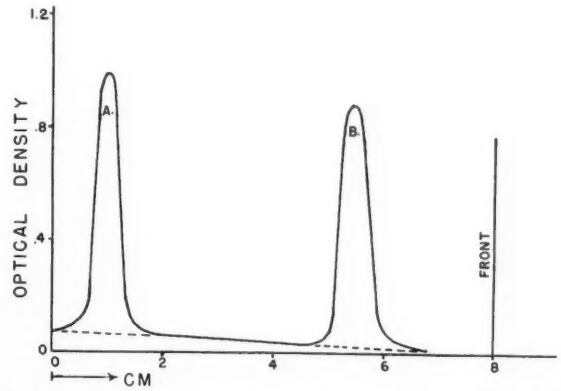


FIG. 7. Ozonization-reduction-thin-layer chromatographic analysis of glyceryl-1,3-dipalmitate-2-oleate and tripalmitin (solvent 15% ethyl ether in Skellysolve F). *A*. Glyceryl residue of glyceryl-1,3-dipalmitate-2-oleate. *B*. Tripalmitin.

Triplicate analyses of this standard mixture, summarized in Table 1, indicate that the average value of three analyses may be expected to vary, generally, less

than 2 percentage units from the individual analyses and from the known composition.

TABLE 1. ANALYSIS OF A MODEL MIXTURE OF TRIGLYCERIDES

Triglyceride Component	Type	Per Cent Composition				
		Found			Average	Known
Tripalmitin	GS_3	23.4	25.4	22.2	23.7	24.8
Glyceryl-1,3-dipalmitate-2-oleate	GS_2U_1	24.6	25.6	22.4	24.2	25.2
Glyceryl-1-palmitate-2,3-oleate	GU_2S_1	30.2	25.2	31.2	28.9	29.0
Triolein	GU_3	21.8	23.8	24.2	23.3	21.1

The analyses of lard and soybean oil are compared with values obtained by others, in Table 2. Some divergence in analysis may be expected because of natural variations in the composition of these oils. Since our results are in the same range of values found by others, they demonstrate that natural fats and oils such as these can be analyzed by this method.

TABLE 2. ANALYSIS OF THE TRIGLYCERIDE TYPES OF LARD AND SOYBEAN OIL

Triglyceride Type	Lard			Soybean Oil		
	Found	Literature Values (4)	(5)	Found	Literature Values (34)	
	per cent			*per cent*		
GS_3	6.7	2.4	2.8	none	0	0.2
GU_1S_2	34.6	28.0	27.4	3.6	3.7	4.3
GU_2S_1	42.7	40.1	54.8	29.3	31.0	29.2
GU_3	16.0	29.5	15.0	67.1	65.3	66.3

DISCUSSION

One of the most important basic principles involved in this method is that the reduction of the ozonides to aldehydes by the Lindlar catalyst (lead-poisoned palladium) is quantitative; other common catalysts, platinum and palladium, for example, give mixtures of alcohols and aldehydes.[1] Since many of the separations are based on relatively fine differences in polarity, production of a mixture of products would be undesirable.

Column chromatographic methods of analysis such as those described by Borgström (17) and Hirsch and Ahrens (35) can also be used for the analysis of the products of ozonization-reduction instead of thin-layer chromatography. Our initial studies were carried out with silicic-acid columns, and quantification was comparable to that obtained with thin-layer silica gel chromatography. However, column chromatographic methods become unwieldy and very time-consuming for the analysis of multiple samples.

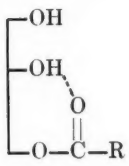
The possibility of transesterification, isomerization, or other spurious reactions occurring during the various stages of the procedure were fairly well eliminated by the clear-cut separations and the lack of streaking of the pure standards and mixtures thereof on the chromatographic plates. Furthermore, identical results were obtained on rechromatographing material recovered from duplicate plates. Recovery of fractions that migrate with the solvent front, for example, for reanalysis with another solvent system is common practice.

Borgström (17) found that 2-monoglycerides were partially isomerized to the 1-isomers during passage through a column of silicic acid, although di- and triglycerides remained unchanged. His report and our inability to separate α- and β-monoglycerides by thin-layer chromatography gave us reason to suspect that perhaps some change occurred during the thin-layer chromatography of these compounds. However, no α-isomer could be detected (21) in the sample of 2-monopalmitin (oxidized with periodic acid to remove the 1-isomer originally present) recovered from a chromatographic plate after being subjected to a normal chromatographic analysis similar to that demonstrated in Figure 2. Thus it seems unlikely that there is any alteration of the structure of monoglycerides during thin-layer chromatography. The inability to separate α- and β-monoglycerides indicates that these compounds have essentially the same degree of polarity. The observation that they have the same partition coefficient (36) is further evidence to this effect. Although the β-isomer contains two primary hydroxyl, and presumably would be more polar than the α-isomer, which contains one primary and one secondary hydroxyl, this is probably nullified, according to Dr.

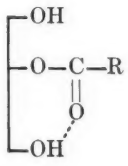
[1] O. S. Privett, C. Nickell, J. A. Schmit, and W. O. Lundberg. Unpublished work.

L. J. Morris,[2] by the effect of hydrogen bonding, as demonstrated below:

α-Monoglyceride



β-Monoglyceride



It may be seen from these structures that each compound has only one free primary hydroxyl group. The bound hydroxyl groups obviously contribute to the polarity of these compounds, but apparently in the bound state the primary and secondary hydroxyl groups do not differ much in their contribution to the total polarity.

At present the method has not been extended beyond the analysis of component mono-, di-, and triglycerides of simple fatty acid composition. However, since a host of fats and oils fall into this category, the method should be valuable to many studies of lipid metabolism and plant lipid chemistry, especially since it offers features not common to other procedures. These may be enumerated as follows: (*a*) It can be carried out on a microscale with an accuracy and precision comparable to existing methods; (*b*) it is based on the separation and analysis of single components, an important feature for further characterization of individual compounds, especially those containing labeled atoms; (*c*) it is relatively fast and simple; (*d*) it permits the estimation of four of the six possible triglyceride types, six of the seven possible diglyceride types, and the four possible monoglyceride types, analyses not now readily performed.

For more general application, the method should be extended to include the determination of the isomers of GU_2S_1 and GU_1S_2, and to glycerides varying widely in their fatty acid constituents, both with regard to chain length and degree of unsaturation. It also would be desirable to combine the above procedure with methodology for the determination of the constituent fatty acids of the separated components. Further development of the method along these lines is in progress.

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[2] Private communication from Dr. L. J. Morris.

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A fluorimetric micro glycerol method and its application to the determination of serum triglycerides

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SUMMARY

A fluorimetric method has been developed for the estimation of glycerol in aqueous solution. It utilizes a series of reactions in which glycerol is heated with *o*-aminophenol in the presence of concentrated sulfuric acid and an oxidizing agent, to form 8-hydroxyquinoline which produces fluorescence on chelation with a divalent metal ion in alkaline solution. Experimental details are given for the estimation of serum triglycerides on phospholipid-free serum lipid extracts. The method can also be used for the estimation of phosphatide glycerol.

Recent interest in fat metabolism has created a demand for a specific routine method for the direct determination of serum triglyceride levels. While indirect methods which are based on the measurement of serum total fatty acids, cholesterol esters, and phospholipids are available, these require calculation of triglyceride levels by difference, and are unsatisfactory mainly because of the assumptions which have to be made, particularly of the proportion of fatty acids available in the phospholipids.

The application of more exact techniques has been rendered possible by the development of column chromatographic methods for the separation of triglycerides from other serum lipid components, but these tend to be too cumbersome and time-consuming for routine use. The recently published method of Van Handel and Zilversmit (1), with minor modifications, has proved very suitable for routine separation of phospholipids from other serum lipid components. Subsequent alkaline hydrolysis of the triglyceride extract according to Carlson and Wadström (2) allows for measurement of the liberated glycerol by the chromotropic-acid method of Lambert and Neish (3).

The micromethod to be described, which has been adapted from a spot test described by Feigl (4), is simple, accurate, and very sensitive. It utilizes a series of reactions based on the Skraup (5) quinoline synthesis, in which glycerol, liberated from triglycerides by alkaline hydrolysis, is heated at 140° with *o*-aminophenol in the presence of concentrated sulfuric acid and an oxidizing agent to form 8-hydroxyquinoline. The fluorescence produced by chelation of 8-hydroxyquinoline with a divalent metal ion in alkaline solution is utilized to provide a quantitative measure of the glycerol, and therefore of triglyceride concentration.

EXPERIMENTAL

Reagents. All reagents and solvents used were analytical reagent grade, with the exception of *o*-aminophenol, of which only a technical grade was available Light petroleum ether (b.p. 30°-60°) was redistilled before use. Diethyl ether and isopropyl ether were freed from peroxides by passage through a column of activated alumina (heated overnight at 170°) just prior to use.

Silicic acid: Silicic acid (Mallinckrodt; 100 mesh, suitable for chromatography) was size graded by sedimentation with distilled water. The fine particles, which constituted approximately 50% of the total, were separated, dried, and activated overnight at 170°.

Arsenic acid solution (0.6%): Fifty g of arsenic pentoxide are dissolved in 100 ml water, allowed to

stand for 3 to 4 days to form H_3AsO_4, and filtered if necessary. One ml of this stock solution is diluted to 100 ml with concentrated H_2SO_4.

o-Aminophenol solution (1.6%): Technical grade *o*-aminophenol is purified by sublimation at 170° in an atmosphere of nitrogen. The sublimed compound tends to oxidize rapidly at this temperature, and it is therefore either recrystallized from, or washed 3 times with, a small volume of isopropyl ether until a colorless solution is obtained when the pure white crystalline compound is dissolved in acetone. This pure product is stable indefinitely if stored in a brown, stoppered bottle. Immediately before use, 0.16 g of the pure *o*-aminophenol is dissolved in 10 ml acetone.

Mg^{++} solution (120 μg/ml): $MgSO_4 \cdot 7H_2O$ (0.12 g) is dissolved in distilled water and made up to 100 ml.

Triolein standards: A stock standard solution is prepared, containing 100 mg triolein (or equivalent amounts of other triglycerides) per 100 ml in chloroform. Dilute standards are prepared, containing 0.2 to 1.0 mg/ml, corresponding to the range 100 to 500 mg triglyceride per 100 ml serum when carried through the procedure.

Procedure. Activated silicic acid (1.2 g) is slurried in a glass-stoppered test tube with 1 ml of isopropyl ether, and 0.3 ml of serum added dropwise with shaking. A further 6.5 ml of isopropyl ether is then added, together with a few glass beads, and the mixture well shaken for half an hour. The silicic acid is allowed to settle, and a 5 ml aliquot of the supernatant extract (≡ 0.2 ml serum) is taken off into a glass-stoppered 15 ml conical centrifuge tube and evaporated to dryness on a hot-water bath using an air blower. One ml aliquots of the triolein standards are similarly evaporated to dryness.

To the dried extract is added 3 to 4 drops ether, 0.5 ml of methanol, and 3 drops of 2% methanolic KOH solution, and the mixture saponified for 30 minutes at 60° to 70°. Two drops of 6% methanolic acetic acid solution are then added, and the mixture evaporated just to dryness on a boiling water bath. Six ml of petroleum ether are added to the hot tube, followed by 0.5 ml of 10 N H_2SO_4, and the tube is stoppered, well shaken, and then centrifuged for 1 to 2 minutes. The petroleum ether layer is carefully pipetted off and discarded. Duplicate 0.1 ml aliquots of the aqueous glycerol phase are then treated as below.

One-tenth ml aliquots of the 1.6% *o*-aminophenol solution are pipetted into a number of test tubes fitted with glass stoppers, and the solvent evaporated, using an air blower. A 0.1 ml aliquot of the serum glycerol extract prepared above is added, followed by 0.4 ml of the 0.6% arsenic acid solution, and the mixture heated in a silicone oil bath at 140° for 15 minutes. The mixture is cooled in ice water, and 1 ml of the Mg^{++} solution is cautiously added with mixing. Five ml of 28% ammonia solution is carefully added, and the tube stoppered and well shaken.

A blank (0.1 ml of 10 N H_2SO_4 solution) and glycerol extracts (0.1 ml), derived as before from 1 ml aliquots of the standard solutions of triolein, are run simultaneously throughout the procedure.[1] After 5 to 10 minutes, aliquots of the above solutions are poured into Farrand fluorometer tubes, and the fluorescence produced by ultraviolet light is measured in a Farrand fluorometer, using aperture 6, with Corning 5874 (primary) and 2424 (secondary) filters. The fluorometer is set at 100% transmission with the highest standard (i.e., 200 μg corresponding to 500 mg/100 ml serum). Where low serum triglyceride concentrations are expected, lower concentration glycerol standards may be used for setting the instrument at 100% transmission, but in this case the blank reading will be higher, as shown in the Figures. It is important that all glassware should be scrupulously clean in order to obtain reproducible results.

RESULTS

The Fluorescence Reaction. The Skraup reaction is commonly used for the synthesis of quinoline derivatives from aromatic amines and glycerol in the presence of concentrated sulfuric acid and an oxidizing agent such as nitrobenzene or arsenic acid. Acrolein, formed by oxidation of glycerol, is probably an intermediate product in the reaction since it will also form quinoline derivatives. The use of *o*-aminophenol as the aromatic amine results in the formation of 8-hydroxyquinoline; however, a large excess of the reagent is required to ensure quantitative reaction with regard to glycerol. This leads to relatively high blanks as well as marked quenching of the fluorescence subsequently produced. Optimum conditions have nevertheless been established in which the influence of these two factors is minimized, and the graph of fluorescence against glycerol concentration is linear for the range 0 to 20 μg glycerol.

Triglycerides such as triolein, tripalmitin, tristearin, and trilinolein have all been found to give quantitative recoveries of glycerol after hydrolysis when checked

[1] Alternatively, glycerol standards, containing 2 to 20 μg of glycerol per 0.1 ml in 10 N H_2SO_4, can be used, since the hydrolysis of the triglyceride is quantitative. Satisfactory results are also obtained with aqueous glycerol solutions.

against glycerol standards. These results have also confirmed, as shown by Carlson and Wadström (2), that glycerol is not lost during the solvent evaporation after saponification. Triolein has been used as the standard for serum triglycerides, and its fluorescence curve is shown in Figure 1.

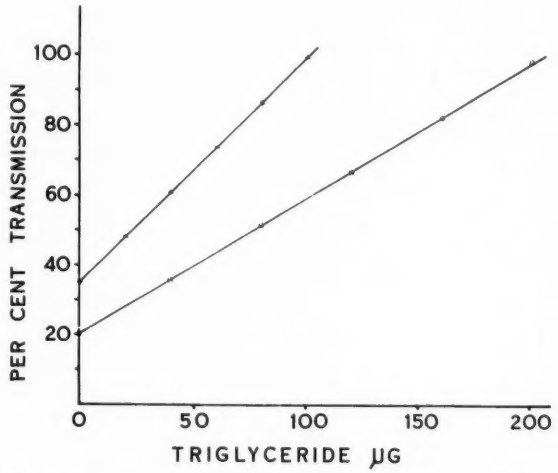

FIG. 1. Relationship between triglyceride concentration and intensity of fluorescence produced by pure *o*-aminophenol.
○——○——○; 200 µg standard set at 100% transmission.
x——x——x; 100 µg standard set at 100% transmission.

The precision of the fluorescence reaction has been determined on duplicate aqueous glycerol extracts derived from 20 different sera. The results indicate an average error of 3.9 mg for the range 17 to 363 mg/100 ml serum.[2] The error will also depend on the sensitivity of the fluorometer, since the range 20% to 100% transmission corresponds to 0 to 200 µg triglyceride when the instrument is set at 100% transmission with the 200 µg standard. As indicated in the procedure and shown in Figure 1, the error can be reduced by approximately one-third by setting at 100% transmission with the 100 µg standard. In this case, the range 35% to 100% transmission corresponds to 0 to 100 µg triglyceride. Since the method utilizes the glycerol liberated from triglycerides, small errors will be introduced by the presence of mono- and diglycerides which can occur up to about 5% to 10% in serum (6).

The influence of a number of serum components on the fluorescence reaction has been investigated. Inositol, glucose, and other hexose sugars do not affect the reaction. Serine, and bases such as choline and ethanolamine, also have no effect. Methanol and ethanol produce interfering fluorescence probably through their aldehydes; however, they are completely removed by evaporation after saponification of the triglycerides. Fatty acids and sterols, when heated with dehydrating agents such as concentrated sulfuric acid, produce interfering fluorescence, and are therefore removed with petroleum ether after saponification. Glycerophosphatides and sphingomyelin interfere in the reaction (see below), and are removed from the serum lipid extracts by silicic acid, as determined by the sensitive phosphorus assay procedure of Chen *et al.* (7). The concentration of free glycerol in serum is small relative to that of triglyceride, and its influence on the fluorescence would therefore be negligible. Moreover, addition of as much as 100 mg of glycerol or glucose to serum prior to extraction with silicic acid and isopropyl ether has had no influence whatsoever on the fluorescence reaction.

It has not been possible to assess directly whether any other serum components alter the serum blank or influence the amount of fluorescence. However, excellent correlation is obtained when the results of the fluorimetric method are compared with those of other procedures described later, and this indicates that any such interference is negligible.

Extraction of Nonphospholipid Serum Lipid Components. In their preparation of phospholipid-free serum extracts, Van Handel and Zilversmit (1) use Doucil and chloroform; however, filtration of the extract is necessary because of the high density of the solvent. Diethyl ether and isopropyl ether, which also extract nonphospholipids from silicic acid, do not require filtration since the silicic acid settles easily from the lower density ethers. Diethyl ether proved to be too volatile and losses were obtained at the shaking stage. These were prevented by the use of isopropyl ether (b.p. 67.5°), which has the additional advantage of having a very low mutual solubility with water.

Elution of all nonphospholipid serum lipid components by the isopropyl ether is quantitative. Cholesterol ester and free cholesterol estimations on the extracts by the method of Sperry and Webb (8), were not significantly different from values obtained on whole serum extracts.

The extraction procedure for triglycerides has been tested by recovery experiments. Triolein was added to serum by two methods: *Method 1.* Chloroform solutions of triolein were added to the stoppered test tubes, and the solvent blown off. Serum (0.3 ml) was added

[2] This has been calculated as the standard error of a single determination, S.D. $= \sqrt{\frac{\Sigma\Delta^2}{2N}}$, where Δ is the difference between two single tests performed on each sample, and N is the number of double determinations.

to the tubes and the mixture shaken. Silicic acid, premixed with a small amount of isopropyl ether, was then added and the mixture slurried with a glass rod. The remainder of the isopropyl ether, together with a few glass beads, was then added, and the mixture well shaken. Aliquots of serum were analyzed with and without the addition of triolein. *Method 2.* Chloroform solutions of triolein were added to larger (5 ml) serum aliquots, the solvent was blown off with N_2, and the mixture was well shaken for half an hour. Aliquots of serum were analyzed before and after the above addition. Recoveries of added triolein varied from 97% to 103% as shown in Table 1.

TABLE 1. RECOVERY OF TRIOLEIN ADDED TO DIFFERENT SERA

Serum	Added	Recovered	Recovered
	μg	*μg*	*per cent*
A (Method 1)	100	100	100
	150	155	103
	200	202	101
A (Method 2)	80	78.5	98
	40	39.6	99
	20	19.4	97
B (Method 2)	50	50.5	101
	100	97	97
	150	147	98
	200	204	102

Triglycerides were estimated fluorimetrically on duplicate aliquots of six serum samples. Results are shown in Table 2. The mean error between duplicate serum analyses was 4.1 mg/100 ml serum, not significantly different from the error on duplicate extracts of the same serum.

Comparison with Other Methods of Triglyceride Analysis. Triglycerides were estimated on 15 sera by the following three methods: (*a*) The proposed fluorimetric procedure. (*b*) By colorimetric estimation of ester groups using a modification (9) of the ferric hydroxamate procedure of Stern and Shapiro (10). This technique was carried out on the extract derived in (*a*) and also on whole sera extracted with Bloor solvent. Free and ester cholesterol were estimated on both extracts by the method described in the previous section (8). The mean molecular weight of the cholesterol ester fatty acids was assumed to be 280. On the whole serum extracts, triglyceride fatty acids were calculated according to the method of Thannhauser and Reinstein (11), serum phospholipids being estimated on the extracts according to the method of Fiske and Subbarow (12). (*c*) By the liberation and electrometric titration at pH 9.0 of fatty acids obtained from serum triglyceride fractions after chromatography of whole serum lipid extracts on silicic-acid columns according to Barron and Hanahan (13).

The results obtained by these methods are shown in Table 3. On the same isopropyl ether extract the fluorimetric method and the ester group method showed an average error of 4.1 mg/100 ml serum, not significantly different from the errors present in each method. Comparison of the fluorimetric and titrimetric methods produces a slightly higher average error of 5.4 mg/100 ml serum, while comparison against the ester group method on whole serum extracts shows the highest average error of 13.0 mg/100 ml serum. The latter method gives lower values than the other methods, probably because of an error in the assumption of the factor (0.69) for the calculation of phospholipid fatty acids (11).

Elution of free fatty acids by the isopropyl ether is also quantitative, and provides a simple method for their extraction free from other acidic serum components. Using larger serum aliquots the free fatty acids have been titrated directly on the isopropyl ether ex-

TABLE 2. ESTIMATION OF TRIGLYCERIDE CONCENTRATION ON DUPLICATE SERUM ALIQUOTS*

Specimen	Triglyceride Concentration (mg/100 ml serum)			
	1st Aliquot		2d Aliquot	
1	41	41.5	42	39.0
	42		36	
2	55	55.0	59	60.5
	55		62	
3	67	69.0	69	69.0
	71		69	
4	108	108.0	114	115.5
	108		117	
5	109	110.0	113	118.0
	111		123	
6	251	254.0	257	261.0
	257		265	

* S.D. of a single determination is 4.1 mg/100 ml serum.

TABLE 3. TRIGLYCERIDE CONCENTRATION BY DIFFERENT METHODS*

Specimen	Triglyceride Concentration (mg/100 ml serum)			
	A	B	C	D
1	82	80	84	78
2	275	265	283	236
3	17	14	20	0
4	160	163	165	150
5	240	233	251	225
6	263	270	273	253
7	122	121	123	118
8	75	73	75	70
9	55	57	63	38
10	363	359	347	323
11	260	264	265	241
12	266	268	277	247
13	327	312	333	315
14	168	166	163	162
15	122	122	125	118

* A: Fluorimetric procedure on isopropyl ether extract.
B: Ester group procedure on same isopropyl ether extract as in A.
C: Titrimetric method.
D: Ester group procedure on Bloor extract of whole serum.
Comparison A/B: S.D. of a single determination = 4.1 mg/100 ml serum.
Comparison A/C: S.D. of a single determination = 5.4 mg/100 ml serum.
Comparison A/D: S.D. of a single determination = 13.0 mg/100 ml serum.

tracts by electrometric titration at pH 9.0 (or with bromthymol blue) using alcoholic KOH. Recoveries of added palmitic and stearic acid have been quantitative. Results have compared favorably with those obtained by other methods.

Normal Values. Normal values obtained by the fluorimetric procedure for the White and Bantu population in South Africa were reported at the First Annual Congress of the Nutrition Society of Southern Africa, in November, 1959, and have recently been published (14). Mean fasting serum triglyceride levels for young White and Bantu males were 86 and 80 mg/100 ml serum, respectively.

DISCUSSION

The procedure recommended consists of a number of independent stages, each of which has been shown to be quantitative and highly selective. The initial extraction with silicic acid and isopropyl ether provides an efficient procedure for the extraction of the nonphospholipid serum lipid components. It has recently been shown by Cheng and Zilversmit (15), using Doucil in the analysis of rat plasma triglycerides, that the particle size of the adsorbent is an important factor governing the complete lysis of the protein-lipid bonds and adsorption of nonlipid serum components. We have similarly found it necessary to employ silicic acid having a particle size finer than 100 mesh, since larger particle sizes lead to low recoveries of the lipid components. Mallinckrodt silicic acid (100 mesh) has therefore been size graded before activation at 170°. We have subsequently used Baker Analyzed reagent grade silicic acid powder, of which not more than 7% is retained on a 100-mesh sieve, and found that recoveries were quantitative, neither the size-grading nor the activation at 170° being necessary.

The procedure for saponification of the triglycerides and isolation of the aqueous glycerol extract is almost identical to that of Carlson and Wadström (2) and the recovery experiments have confirmed that glycerol is not lost during evaporation of the methanol after saponification. For the acidification, 10 N sulfuric acid was used in order to maintain the water content of the final reaction mixture at a minimum.

As mentioned before, the fluorescence reaction is subject to a fairly high reagent blank produced by the large excess of *o*-aminophenol. It is therefore essential that the reagent be as pure as possible and absolutely colorless before use. The amount of oxidizing agent present is not as important a factor. The production of fluorescence with Mg^{++} ions will be affected only by a very large excess of the latter, which may cause precipitation of the Mg^{++}-8-hydroxyquinoline complex.

In attempting to purify the technical grade *o*-aminophenol (Kodak) by recrystallization from ether, a deep-red compound was isolated from the mother liquor after chromatographic separation on a silicic-acid column according to the following procedure: 50 g of technical grade *o*-aminophenol was refluxed for 1 hour with 500 ml of diethyl ether. Then 500 ml of petroleum ether (b.p. 30°-60°) was added, and the mixture cooled at −15° for half an hour. The residue was filtered off, and the clear supernatant was concentrated to a small volume (± 20 ml), and added to a silicic-acid column (100 g; previously activated at 170° and prepared in a 50:50 ether:petroleum ether mixture). Elution was carried out with this solvent mixture (which removed dissolved *o*-aminophenol) until no residue was obtained on evaporation of an aliquot of the eluate. Elution was then continued with pure ether, and a deep-red band began to move down

the column. The eluate containing this fraction was collected and evaporated to dryness under N_2 (yield: approximately 0.5 g). This impurity has a profound fluorescence quenching effect when added back to pure *o*-aminophenol. On its own, however, it proved to be a highly sensitive reagent for the quantitative estimation of glycerol. Four-tenths ml of a 0.0125% solution of this compound freshly prepared in the 0.6% arsenic acid solution, when added to the 0.1 ml glycerol extract and treated according to the procedure outlined above, produced an intense red fluorescence and a much lower blank reading (Fig. 2). In addition, the

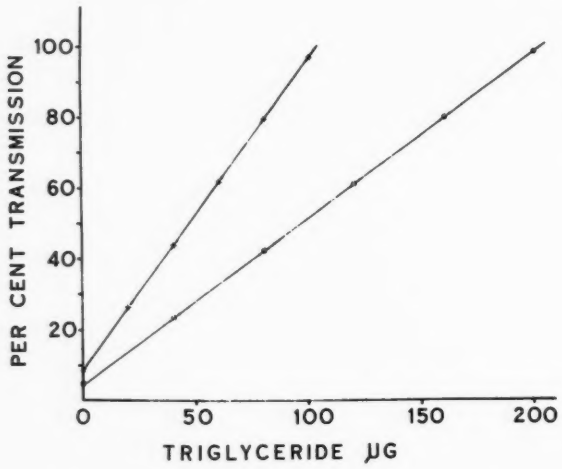

FIG. 2. Relationship between triglyceride concentration and intensity of fluorescence produced by red impurity obtained from technical *o*-aminophenol.
o——o——o; 200 µg standard set at 100% transmission.
x——x——x; 100 µg standard set at 100% transmission.

fluorescence produced in the ammoniacal solution did not require the presence of a divalent metal ion. The nature of this compound is not known, but current investigations suggest a highly polymerized product with the required polar reactive groups, since removal of either glycerol or the oxidizing agent from the reaction mixture did not produce fluorescence.

Application to the Estimation of Phosphatide Glycerol. The proposed method is particularly suited to the determination of glycerol in glycerophosphatides. Lecithins, cephalins, inositol phosphatides (containing inositol instead of a base in the glycerophosphatide), and lysolecithins obtained by fractionation of serum phospholipids on silicic-acid columns according to Hanahan *et al.* (16) have all given 1:1 glycerol:phosphorus molar ratios when analyzed for glycerol content according to the above procedure. The mild alkaline hydrolysis recommended in the procedure readily deacylates the fatty acid moiety of the glycerophosphatide resulting in the production of an equilibrium mixture of α- and β-glycerophosphate esters (17). It would appear, therefore, that both of these esters react quantitatively in the fluorescence reaction.

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Measurement of lipoprotein lipase activity in post heparin plasma: description of technique*

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SUMMARY

A method has been described for the quantitative assay of lipoprotein lipase activity in post heparin plasma. The conditions of the assay were subjected to critical study. The technique described permits the reaction to proceed in the test tube as a zero order reaction. It is believed that this technique will permit accurate quantitative study of lipoprotein lipase activity in different groups of patients. The rate of lipolysis was compared with the rate of clearing of lipemic plasma. The ratio of the rate of lipolysis to the rate of clearing was quite variable in normal subjects. This suggests that the use of the clearing technique to study lipoprotein lipase activity in different groups of patients, or under different experimental conditions, may give misleading results.

In 1943 Hahn (1) reported the rapid *in vivo* clearing of alimentary lipemia following the injection of heparin. In 1950 Anderson and Fawcett (2) demonstrated that plasma obtained after heparin injection contained a factor which cleared lipemia *in vitro*. Since then there have been several hundred studies of the clearing of lipemia by heparin-induced clearing factor. In 1955 Korn (3) established that "clearing factor" is an enzyme, lipoprotein lipase, that splits the triglyceride moiety of chylomicrons and low density lipoproteins to form free fatty acids (FFA) and glycerol. The characteristics of this reaction have been studied by numerous investigators and are summarized in several recent reviews (4, 5). Lipoprotein lipase has been extracted from heart, adipose tissue, lung, and other tissues. Without heparin, the enzyme can be detected in the plasma only in negligible amounts, but the plasma level rises markedly immediately after the intravenous injection of heparin.

When post heparin plasma is incubated with suitable substrates—lipemic plasma, chylomicron suspensions, thoracic duct lymph, or an activated artificial fat emulsion—there is a striking decrease in turbidity of the incubation mixture. It has been shown that under appropriate conditions the clearing proceeds at a constant rate and is proportional to the concentration of the enzyme. The simplicity of this phenomenon has encouraged investigators to use the measurement of optical density as an indication of lipoprotein lipase activity.

The absorbancy of a complex mixture of plasma and emulsified fat is dependent upon the scattering of a beam of light passed through it. The scattering of light by such a mixture is determined principally by the size and number of particles. The particle size is affected by many variables, including chemical composition, electric charge of the particulate matter, and protein distribution. The change in plasma turbidity produced by a given change in plasma triglyceride varies among individuals.[1] Thus plasma turbidity is affected by many incompletely understood physicochemical phenomena, and quantification of lipolytic activity based on rates of decrease in absorbancy might be misleading. Baeder *et al.* (6) have reported *in vitro* clearing of lipemic dog plasma without the occurrence of lipolysis. Evidence is now presented that a decrease in absorbancy does not bear a consistent relationship to lipase activity, and in a related study it has been shown that misleading conclusions can indeed be reached by use of the turbidity technique.[2]

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[1] F. Kern, Jr., B. Sanders, G. McDonough, and L. Steinmann. In preparation.

[2] F. Kern, Jr., B. Sanders, H. Singh, and L. Steinmann. In preparation.

It is believed that a quantitative chemical technique which measures one of the products of lipolysis provides more accurate and useful data.

The reaction catalyzed by lipoprotein lipase as demonstrated by Korn (7) is shown below:

$$\text{Triglyceride} + \text{Protein} \xrightarrow{\text{Lipoprotein lipase}} \underset{\downarrow \text{Albumin}}{\text{Free Fatty Acids}} + \underset{\downarrow Ca^{++}}{\text{Glycerol}}$$

Triglyceride is not a substrate for this enzyme until it has been activated by protein. When mixed with plasma, the triglyceride is activated very rapidly.

FFA are transported bound to serum albumin and they combine with albumin *in vitro* as well (Ca_{++} also exhibits some affinity for these anions). Havel and Fredrickson (8) and Laurell (9) have shown that the half life of FFA *in vivo* is about 2 minutes. Because of this rapid turnover, albumin is normally available to bind the FFA produced *in vivo*, but this is not necessarily true *in vitro*. Since unbound FFA inhibit the lipolysis catalyzed by lipoprotein lipase, it is essential that an adequate amount of albumin be present in the *in vitro* assay. (10)

The requirements of a satisfactory *in vitro* assay of this enzyme are as follows: (*a*) the substrate should be a standardized artificial fat emulsion without inhibitors; (*b*) abundant fatty acid acceptors must be available and optimum conditions for the binding of fatty acids must be achieved; (*c*) the reaction should proceed with zero order kinetics during the period of measurement; (*d*) the chemical technique of measuring the end product must be sensitive, reproducible, and feasible technically for a large number of simultaneous determinations.

Korn's technique for measurement of tissue lipoprotein lipase activity has been modified for use with post heparin plasma so that these criteria are met.

METHODS

Nineteen studies were done on 14 normal medical students and hospitalized patients not acutely ill. All experiments were done after an overnight fast. After drawing a sample of blood, 50 mg of heparin (heparin sodium, 100 mg/ml, Upjohn) was administered intravenously. Exactly 20 minutes later the post heparin sample was collected. Nine ml of blood was mixed with 1 ml of 3.2% sodium citrate and immediately placed in an ice water bath of 0-1°. Within 2 hours the plasma was separated by centrifuging at 0-4° at 1500 × *g* for 10 minutes. In 8 of the 19 studies the post heparin plasma was used for simultaneous measurement of lipoprotein lipase activity by both the chemical and turbidity techniques, and in 11, only the chemical determination was made. The 50 mg dose of heparin and the 20-minute post heparin interval were chosen arbitrarily.

For each 1.0 ml of plasma the incubation mixture contained 1.0 ml of 0.05 M tris buffer (pH 8.5) (Sigma 7-9 tris [hydroxymethyl] amino methane); 100 mg (0.4 ml of a 25% solution) bovine albumin (Fraction V powder, Armour) dissolved in distilled H_2O and pH adjusted to 8.5; and 0.1 ml of the substrate, a 15% fat emulsion. In the technique adopted for use, the substrate used was Ediol®,[3] a commercial 50% coconut oil emulsion diluted with water to a 15% emulsion. In all experiments, except as otherwise indicated, the above reaction mixture was used. Ediol® was compared with Lipomul—I.V.®, a commercial 15% cottonseed oil emulsion, and a specially prepared 15% olive oil emulsion.[4] Some experiments were done using human serum albumin (Cutter) as the fatty acid acceptor, but this proved to be unsatisfactory (see below). Duplicate 0.25 ml aliquots of the reaction mixture were incubated for 10, 20, 30, and 60 minutes and compared with the unincubated samples. In all studies incubation mixtures without added albumin were run simultaneously, but they were assayed usually only at 1 hour, and compared with the glycerol produced at 1 hour in the presence of added albumin.

The albumin palmitic acid complex used in some experiments was prepared by the technique described by Fillerup *et al.* (11). The palmitic acid-albumin ratios were calculated and were not exact since the 0.05 mole fatty acid per mole of albumin already present in bovine serum albumin (Fraction V) was ignored (12).

All plasma samples were kept cold until they were added to the incubation mixture, which was immediately placed in a water bath at 37°. At the same time,

[3] Ediol® was supplied by Schenlabs Pharmaceuticals.

[4] Lipomul I.V. and the olive oil emulsions were kindly provided by Dr. J. B. Lawson of the Upjohn Company.

the unincubated sample was deproteinized. At the various time intervals, incubation tubes were withdrawn, placed in an ice bath, and immediately deproteinized with 2 ml of 0.1 N H_2SO_4 and 0.25 ml 25% sodium tungstate and centrifuged. Glycerol was determined essentially by Korn's modification (3) of the method of Lambert and Neish (13). Deproteinized plasma (0.5 ml) was measured into a 125 × 16 mm Pyrex tube containing 0.5 ml 1.0 N H_2SO_4. H_2O (0.5 ml) was used for the reagent blank and 0.5 ml of a glycerol solution (containing 8 μg glycerol) was used as standard. The tubes were transferred from ice water to a 20° water bath, and 0.1 ml .05 M $NaIO_4$ was added to each tube at the rate of 8 tubes per minute. Exactly 5 minutes after the addition of the periodate, 0.1 ml 0.5 M Na arsenite was added, following the same rate and sequence. After disappearance of the yellow color, 9 ml chromotropic acid solution[5] was added; tubes were covered with marbles and placed in a covered boiling bath for 30 minutes. After cooling in ice water, samples were read against the reagent blank at 570 mμ in a Coleman Junior Spectrophotometer in 15 ml cuvettes. Results were calculated as follows:

$$\frac{\text{Absorbancy test} - \text{absorbancy unincubated sample}}{\text{absorbancy standard}} \times \mu\text{g glycerol of standard} \times$$

$$\text{dilution of plasma in incubation mixture} \times \text{dilution of incubation mixture by deproteinization} = \mu\text{g glycerol/ml plasma}$$

To 10 × 75 mm Coleman photometric cuvettes were added 0.4 ml 25% albumin, 0.3 ml 1.5% coconut oil (Ediol®), and 1.3 ml 0.05 M tris buffer. These tubes were kept at 0-4° and 1.0 ml of the post heparin plasma was added. The tubes were then placed in a 37° water bath and a preincubation period of 4 minutes was allowed before the initial absorbancy reading, which was approximately .600. The readings were made in a Coleman Junior Spectrophotometer at 700 mμ wave length using water as a blank. Following the initial reading, the tubes were removed from the water bath and absorbancy was measured at 10-minute intervals, 30 seconds being required for each reading.

Critique of Method: The data obtained using the technique described are shown in Figure 1. Zero order kinetics were achieved for 30 minutes in the presence of added albumin.

Experiments comparing Ediol® with Lipomul and with the olive oil emulsion showed that the different substrates were hydrolyzed at different rates. It was possible for the reaction to proceed at a constant maximum rate only with Ediol®, the coconut oil emulsion. Other experiments demonstrated that smaller amounts of Ediol® did not provide sufficient substrate for zero order kinetics during the reaction catalyzed by the amount of enzyme usually present.

When post heparin plasma was diluted with preheparin plasma in varying concentrations, it was found that there was a linear relationship between glycerol production and the amount of enzyme present, so long as the reactions were proceeding at a maximum rate (Fig. 2). This re-emphasizes the importance of following the reaction closely so that the velocity is measured at its maximum, since a falling-off of glycerol production is noted in this system when the plasma has a very high activity.

It appeared logical to use human serum albumin as the supplementary fatty acid acceptor in the reaction mixture. When it was used, however, the results suggested that an inhibitor was present, and when compared with bovine albumin (Fraction V powder), it was found that the latter was invariably associated with a greater glycerol production. It was suspected that this difference might be the result of inhibition by stabilizers (sodium caprylate and sodium acetyltryptophanate)[6] present in the human albumin preparation. When these stabilizers were added to the bovine albumin preparation, significant inhibition of lipolysis was observed.

The importance of the amount of albumin in the reaction mixture is demonstrated in Figure 3. There was a progressive increase in reaction rate as the amount of added albumin was increased from none to 150 mg per ml of plasma. In this particular experiment even 150 mg of albumin was not sufficient to maintain a straight line for 1 hour. It may be important to note that the subject for this experiment was a male medi-

[5] Chromotropic acid was prepared by adding 6 volumes concentrated H_2SO_4 to 3 volumes of distilled water. After cooling, 1 volume of a 1% aqueous solution of chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulfonic acid disodium salt, practical grade obtained from Eastman Organic Chemicals) was added. The solution keeps for two weeks stored in a brown bottle at room temperature.

[6] Supplied by Dr. E. B. McQuarrie, Cutter Laboratories.

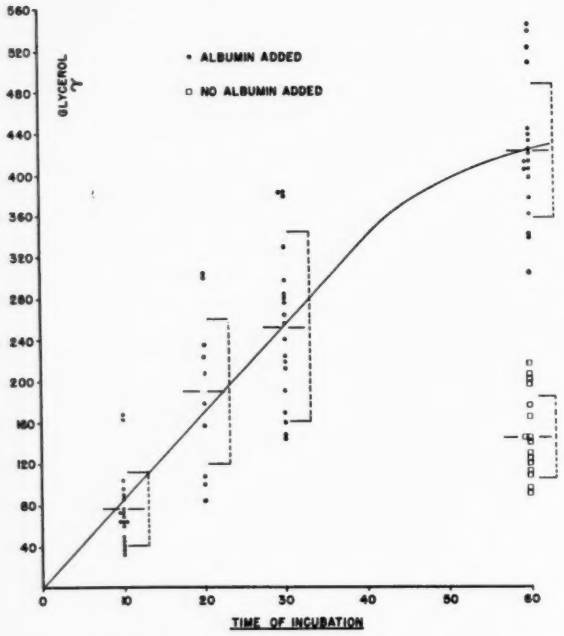

FIG. 1. Lipoprotein lipase activity. Glycerol production as a function of time in 19 normal subjects.

cal student on a voluntarily imposed low calorie, low fat diet.

It was found that 100 mg albumin per ml plasma provided adequate fatty acid acceptor in subjects whose serum albumin was 4.0 gm%. In all of these studies the patient's total serum protein was measured by the $CuSO_4$ technique and the albumin was determined by paper electrophoresis. Sufficient bovine albumin was added to the reaction mixture to bring the total albumin concentration to 140 mg per ml plasma.

Robinson and French (14) reported that when FFA were present in excess of a 6:1 molar ratio to albumin, lipolysis was inhibited. Figure 4 shows an experiment in which enzyme activity was compared using no added albumin, 100 mg of albumin as a 3:1 palmitate-albumin complex, 100 mg of albumin as a 6:1 palmitate-albumin complex, and 100 of unaltered bovine albumin. It is clear that the reaction rate using the 6:1 fatty acid-albumin complex was the same as the reaction rate without any added albumin, and that the 3:1 fatty acid-albumin complex accelerated the rate only about one-half as much as the albumin alone. It therefore appears that the mechanism of action of albumin is solely that of binding the fatty acids released from the triglycerides, thereby preventing their inhibitory effect upon the reaction.

The effect of pH on the kinetics of the reaction is shown in Figure 5. In the absence of added albumin, the reaction proceeds at a more rapid rate at pH 8.5 than at pH 7.5, as shown by Korn (3). The most important point to note, however, is that when additional albumin is made available, there is a very marked increase in reaction rate as the pH is increased. These observations suggest that pH 8.5 is optimum for lipoprotein lipase activity, and that at pH 8.5 the ability of albumin to combine with FFA is greatly enhanced. It was not possible to raise the pH higher than 9.0 with tris buffer. A higher pH might have denatured the proteins and inhibited the reaction. Phosphate buffers were tried but could not be used because the phosphate interfered with the glycerol assay. Using 0.05 M tris buffer (pH 8.5) and the standard reaction mixture as described, it was shown by means of a con-

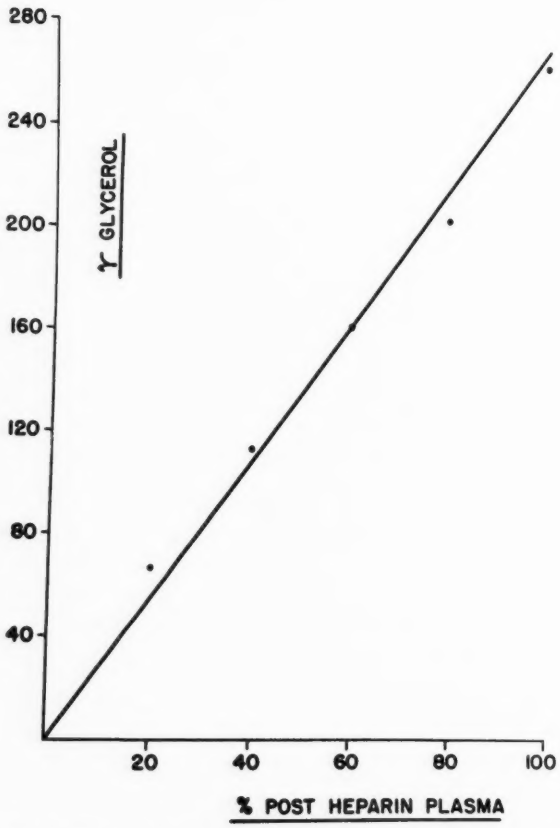

FIG. 2. Lipoprotein lipase: variation in enzyme concentration. The abscissa represents the relative amounts of post heparin plasma mixed with pre-heparin plasma from the same subject. Glycerol production was measured after a 20-minute incubation.

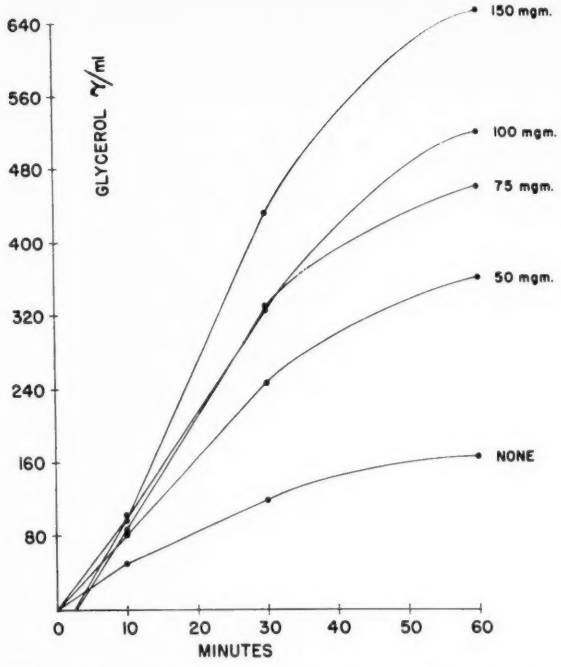

FIG. 3. Lipoprotein lipase activity: effect of added albumin.

tinuous recording of pH that the pH fall during 1 hour of incubation did not exceed 0.6 pH units.

In most experiments the fall in absorbancy was linear for 20 to 30 minutes. A straight line was drawn through this period of most rapid fall and the slope (k) was calculated in terms of Δ absorbancy per ml plasma per hour, as described by Baker (15).

RESULTS

The results of these studies are shown in Table 1. The mean glycerol production was 422 gamma glycerol per ml plasma per hour with added albumin, in contrast to 143 without added albumin. The variation in speed of the reaction in this group of patients is apparent. In almost all experiments the reaction rate was constant during the first 30 minutes of incubation, and in those patients with less enzyme, it continued at a constant rate for 60 minutes. It is to be noted, however, that when the initial reaction rate was very rapid, glycerol production slowed sometime between 20 and 30 minutes. This is easily seen by comparing the measured glycerol produced at 60 minutes with the rate of glycerol production calculated from the straight-line portion of the slope (Table 1). Figure 1 illustrates some of these findings.

Table 1 also shows the comparison between the rate of the reaction as determined by the quantitative chemical technique and by the turbidity technique. In every experiment the rate of glycerol production was proportional to the rate of turbidity decrease (Fig. 6).

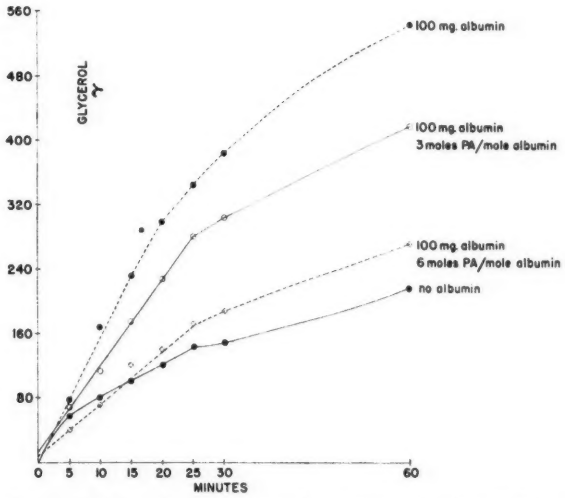

FIG. 4. Lipoprotein lipase activity: effect of albumin-fatty acid complex. This shows the inhibitory effect of prior complexing of palmitic acid with the albumin.

It is apparent, however, that although these two types of measurement bear a definite relationship to each other in the individual patient, there was marked variation from patient to patient. This variability is expressed mathematically in Table 1 as the ratio of the k for glycerol production to the k for Δ absorbancy. The ratio varied from 0.75 to 1.57.

DISCUSSION

A method for measuring the *in vitro* lipolytic activity of lipoprotein lipase has been described. With the amount of enzyme produced by 50 mg of heparin, zero order kinetics are maintained *in vitro* for at least 30 minutes in most subjects. The need for a large amount of albumin to bind the FFA produced has again been demonstrated and the importance of maintaining the optimum pH for maximum enzyme activity and for maximum albumin binding of FFA has been shown. The measurement of an end product of the lipoprotein lipase activity under conditions permitting zero order

kinetics permits quantitative interpretation of the lipolytic potential of the enzyme *in vivo*. For example, rough calculation of the amount of triglyceride split in a 70 kilogram man with average *in vitro* activity during one hour following 50 mg of heparin (assuming the same level of activity for 1 hour) shows that 14.4 g of triglyceride could be hydrolyzed in the plasma.[7]

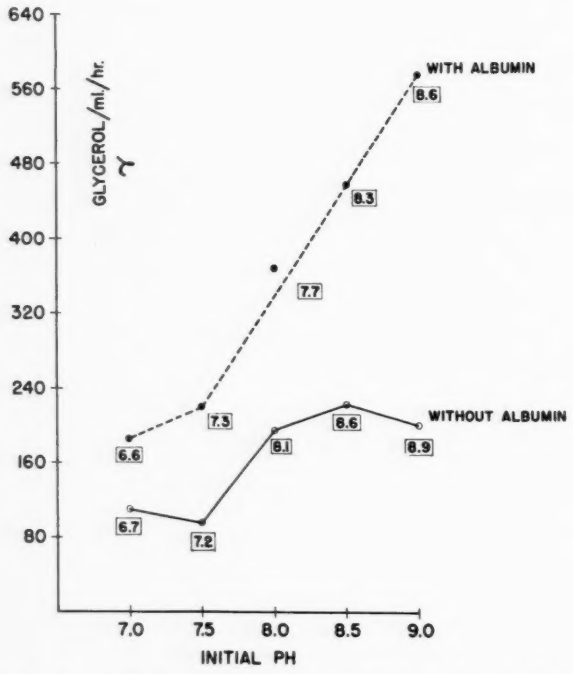

FIG. 5. Effects of pH on lipoprotein lipase activity. The boxed numbers indicate the final pH after incubation for one hour.

TABLE 1

No.	Subject: Diagnosis, Age, Sex	Glycerol/ml Plasma/hour: Without Albumin	With Added Albumin: Measured	With Added Albumin: k Calculated	Clearance k	Glycerol k Clearance k
1.	Normal-35-F	175	305	305		
2.	Pleural effusion-35-F	110	338	338		
3.	Rheumatoid arthritis-43-F	144	342	342		
4.	Arsenic poisoning-73-M	108	359	359		
5.	Cerebral vas. accident-73-M	94	377	560	0.56	1.0
6.	Convalescent pneumonia-53-M	123	395	448	0.60	0.75
7.	Gastric ulcer-50-M	127	405	405	0.29	1.39
8.	Normal-23-M		405	560		
			413	700		
			432	598	0.39	1.53
			438	572		
		165	522	688		
		217	543	906		
9.	Gastric ulcer-47-M	206	413	413		
10.	GI bleeding cause undet. 40-M	119	420	512	0.35	1.46
11.	Duodenal ulcer-25-M	200	425	425		
12.	Pyelonephritis-56-M	92	444	560	0.53	1.06
13.	Recovered hepatitis-31-M	129	508	658	1.16	0.56
14.	Gastric ulcer-53-M	144	535	912	0.58	1.57
Mean		143.5	422	540	0.56	1.16

The rate of decrease in absorbancy (clearance k) was calculated from the straight-line portion of the curve extrapolated to 1 hour. The glycerol k was calculated in the same manner.

The rate of lipolysis observed in these studies is rapid because the reaction was allowed to achieve its maximum rate. If large amounts of heparin are used, it is possible that more enzyme would be mobilized, necessitating more substrate and more albumin in proportion to the amount of plasma in the reaction mixture.

[7] An *in vitro* assay of 550 gamma of glycerol per ml plasma per hour is equivalent to 6 μM glycerol per ml plasma per hour. Assuming that each mole of glycerol represents a mole of triglyceride (since a small part of the glycerol measured is monoglyceride, this assumption leads to the slight overestimation of triglyceride). Then in 3000 ml of plasma 18,000 μM of triglyceride could be split. Using an average molecular weight of triglyceride with long-chain fatty acids, 800, 18,000 μM is equivalent to 14.4 g of triglyceride.

The relationship between lipolysis, as measured chemically, and the difference in absorbancy of the plasma is of considerable interest and of great practical importance. In the individual patient these measures are proportional to each other; that is, as lipolysis proceeds, the absorbancy decreases. This observation has been made by many others; of equal importance, however, is the finding that the ratio between lipolysis and turbidity decrease in different patients is highly variable. It can be readily seen from Figure 6 that it is impossible to predict the rate of lipolysis from the rate of decrease in absorbancy. This finding raises considerable doubt about the meaning of studies of post heparin lipoprotein lipase activity in different age

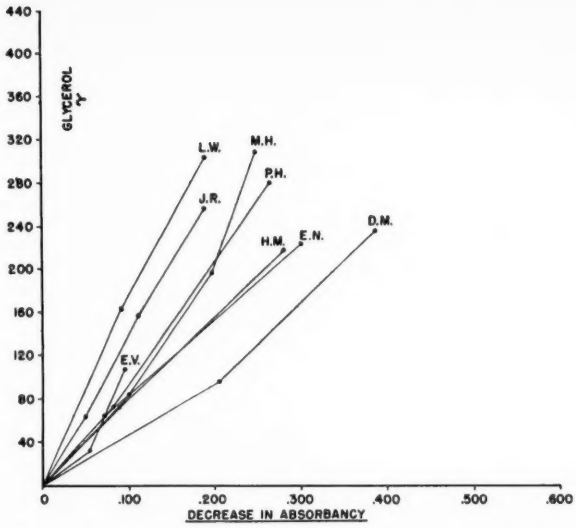

FIG. 6. Lipoprotein lipase: comparison of glycerol production with decrease in absorbancy. Glycerol production plotted against clearing of turbidity in 8 normal subjects.

groups and disease states when the turbidity technique was employed as the means of measuring the enzyme activity.

Studies of the role of certain organs of the body in the production and destruction of this enzyme have also been based upon the lipemic clearing action rather than chemical measures of lipolysis. For example, the liver is generally believed to inhibit lipoprotein lipase activity, but most studies leading to this conclusion have measured decrease in absorbancy as the major index of activity (16 to 19). Evidence collected in our laboratory concerning the role of the liver, using both the chemical measure of lipolysis and the turbidity technique, strongly suggests that rapid clearing *in vitro* of post heparin plasma of cirrhotic subjects is not adequately accounted for in terms of lipolysis.[8]

[8] See footnote 2.

The results of the studies reported here indicate that measuring the decline in absorbancy can be useful to detect the presence or absence of post heparin clearing factor, but it should not be used to quantify or compare the rates of lipoprotein lipase activity under different experimental or clinical conditions.

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The use of pancreatic lipase for determining the distribution of fatty acids in partial and complete glycerides

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SUMMARY

The method of digestion with pancreatic lipase for determining the position of fatty acids in triglycerides has been modified to increase its accurary and ease of applicability. Moreover, by acylation of partial glycerides with a "marker" fatty acid, the method can be used for determining the structure of mono- and diglycerides. Evidence is presented which demonstrates that acylation of partial glycerides with fatty acid chlorides can be carried out without causing rearrangement of the partial glycerides.

The demonstration that pancreatic lipase hydrolyzes specifically the fatty acids esterified with the primary hydroxyl groups of glycerol (1) served as the basis for a method to determine the distribution of fatty acids in a triglyceride (2, 3, 4). Considerable experience with this method has led to a number of refinements in the technique. Since interest in the chemistry and metabolism of glycerides of specific structures has become widespread, it seems desirable to report these refinements in technique. Moreover, by acylation of partial glycerides and hydrolysis of the resulting triglyceride with pancreatic lipase, it is also possible to determine the structure of mono- and diglycerides.

METHODS

Determining the Structure of Triglycerides. The steps in determining the structure of a triglyceride are: (*a*) digestion of the triglyceride with pancreatic lipase, (*b*) isolation of the monoglycerides from the products of digestion, and (*c*) determination of the fatty acid composition of these monoglycerides and the original triglyceride. Since the initial steps in the hydrolysis of a triglyceride by pancreatic lipase are the splitting-off of the fatty acids esterified with the primary hydroxyl groups of glycerol, the fatty acids present in the monoglycerides after the digestion will be those that occupied the 2-position in the triglyceride. The hydrolysis of the primary ester groups is random, showing neither fatty acid specificity (2) nor stereospecificity (5).

The conditions of digestion have been somewhat simplified from that originally reported (1). As presently carried out, the digestion mixture consists of 0.5 g of triglyceride, 9.0 ml of 1.0 M tris (hydroxymethyl) aminomethane adjusted to a pH of 8.0, 0.5 ml of a 45% aqueous solution of calcium chloride, 0.2 ml of a 1% aqueous solution of bile salts,[1] and 80 mg pancreatin[2] suspended in 1 ml of tris buffer. The pancreatin suspension is prepared immediately before use. The digestion is carried out at 40° with continuous rapid agitation. Digestion is continued, usually from 15 to 30 minutes, until approximately 60% of the esterified fatty acid has been hydrolyzed. The time of digestion necessary to obtain this degree of hydrolysis is determined with each batch of pancreatin.

At the end of the hydrolysis period 5 ml of dilute hydrochloric acid 1:1 (v:v) and 15 ml of ethanol are added. The lipids are then recovered by extraction with ethyl ether. The ethyl ether solution is washed three times with water, dried with sodium sulfate, and the solvent removed under vacuum. The resulting lipids are dissolved in benzene. The monoglycerides are isolated from these by chromatographing on silica gel by the method of Quinlin and Weiser (6), except that

[1] Bile Salts No. 3. Difco Laboratories, Detroit, Mich.
[2] Lipase (Steapsin). Nutritional Biochemical Corp., Cleveland, Ohio.

300 mm × 10 mm columns containing 10 g of silica gel are used. The fractions are eluted with 90 ml portions of solvent. The monoglyceride fraction is obtained from the column in solution in ethyl ether and contains about 5% of free fatty acid (FFA). To remove these FFA, an ethyl ether slurry containing approximately 5 g of Dowex 2-X8, 20-50 mesh anion exchange resin in the hydroxyl form is added. After filtering, the solvent is removed under vacuum. The fatty acids of the monoglyceride fraction and the original triglyceride are characterized by appropriate methods. The weight per cent of a particular fatty acid esterified with the 2-position will be equal to:

$$\frac{\text{per cent of the particular fatty acid in the monoglyceride formed}}{\text{per cent of the particular fatty acid in the original triglyceride} \times 3} \times 100.$$

During enzymatic hydrolysis, some of the fatty acids esterified in the 2-position of the triglyceride are split off. Whether this is due to an actual hydrolysis at the 2-position or whether the fatty acid migrates to one of the primary hydroxyl groups prior to hydrolysis is not known. Such splitting is indicated by the results shown in Table 1. Here a sample of cottonseed oil was hydrolyzed for various time periods and the iodine values of the FFA and of the monoglycerides were determined. The iodine value of the monoglyceride fatty acids remained constant but there was a gradual increase in the iodine value of the FFA. This increase in iodine value is the result of the splitting off of the acids esterified at the 2-position of glycerol, since in cottonseed oil the fatty acids occupying the 2-position are more unsaturated than those in the 1- and 3-positions (3).

TABLE 1. IODINE VALUE OF THE MONOGLYCERIDES AND FREE FATTY ACIDS RELEASED FROM COTTONSEED OIL BY PANCREATIC LIPASE AS A FUNCTION OF TIME

Time of Hydrolysis	Fatty Acids of the Monoglyceride	Free Fatty Acids
minutes		
15	154.9	87.8
20	155.4	92.1
30	155.0	93.5

Determining the Structure of Partial Glycerides. The principle of this determination depends on: (*a*) esterifying the partial glyceride with a "marker" fatty acid, (*b*) digesting the resulting triglyceride with pancreatic lipase, (*c*) isolating the monoglycerides from the products of digestion, and (*d*) determining the content of marker fatty acid in the monoglycerides resulting from digestion with pancreatic lipase. The enzymatic digestion and the isolation of the monoglycerides formed are carried out as already described in the section on Determining the Structure of Triglycerides. The marker fatty acid found in the monoglycerides present after digestion with pancreatic lipase will be proportional to the free hydroxyl groups in the 2-position of the original partial glycerides, with allowance being made for differences in molecular weight among the fatty acids. The marker fatty acid can be an isotopically labeled fatty acid or any fatty acid not present in the original partial glyceride. The method chosen for determining the content of marker fatty acid will depend on the nature of the fatty acids present in the original partial glyceride and the marker fatty acid used. For example, if the partial glyceride contains only saturated fatty acids, an unsaturated acid can be used as the marker, in which instance simply determining the iodine value of the monoglycerides formed by lipase hydrolysis will suffice.

The acylation of the partial glycerides with the marker fatty acid is carried out as follows. Approximately 0.6 g of monoglyceride or 0.9 g of diglyceride is dissolved in 10 ml of water-washed, distilled, and dried chloroform. An amount of pyridine equal, on a molar basis, to the amount of acid chloride to be used is added, followed by one and one-half times the amount of acid chloride which is necessary for esterification. The mixture is allowed to stand at room temperature for 3 days. As will be shown subsequently, the partial glycerides are completely esterified with no isomerization having taken place. The reaction products are dissolved in ethyl ether, washed twice with water, three times with 1% aqueous hydrochloric acid, four times again with water, dried with sodium sulfate, and finally the solvent is removed under vacuum. The washing steps are for the purpose of removing the pyridine and converting any remaining acid chloride to the free acid.

The lipids recovered from the acylation procedure consist of about 85% triglyceride and 15% FFA. The yield of triglyceride is from 90% to 100% of theory. The complete removal of this amount of FFA would be laborious. However, no attempt is made to remove this, since further steps in the procedure depend only on the glyceride fraction. To demonstrate that this residual FFA does not interfere with the method, samples of

1,2- and 1,3-dipalmitin[3] were esterified by this procedure. A portion of the resulting triglycerides was purified further by crystallization to an acid value of less than 1. The crude and recrystallized triglycerides were hydrolyzed with pancreatic lipase and the distribution of the marker fatty acid determined. In this instance, oleic-1-C^{14} acid was used. Samples were analyzed by counting in a Tri-Carb liquid scintillation spectrometer. The results are given in Table 2. It is apparent that this presence of FFA in the crude triglycerides did not alter the results.

TABLE 2. COMPARISON OF THE STRUCTURE OF DIGLYCERIDES AS DETERMINED ON CRUDE OR RECRYSTALLIZED TRIGLYCERIDES RESULTING FROM THE ACYLATION OF THE DIGLYCERIDES

Diglyceride Source	Per Cent 1,3 Isomer as Determined on:	
	Crude Triglycerides	Recrystallized Triglycerides
1,3-Dipalmitin	91	92
1,2-Dipalmitin	5	3
Mixture 1,2- and 1,3-Dipalmitin	60	61

For this method to be successful, it is necessary that there be complete acylation of the partial glycerides with the marker fatty acid and no migration of the fatty acid groups on the glyceride. Acylation was shown to be complete in that the glycerides isolated at the end of the acylation step had a hydroxyl value of zero, and acylation with radiotagged fatty acid yielded a glyceride which contained an amount of tagged acid theoretically correct for the formation of triglycerides.

Fischer and Baer (9) have reported that migration of fatty acids does not occur during acylation of monoglycerides with fatty acid chloride. Their conclusion was based on an unchanged optical activity and the content of 1-monoglyceride following exposure of an optically active 1-monoglyceride to pyridine. To demonstrate that this is also true of diglycerides, the following study, in which acylation occurred in two steps, was carried out. Samples of 1,2- and 1,3-dipalmitin were treated separately with acid chloride according to the procedure described here, except that for each mole of diglyceride one-half mole of carboxyl-tagged oleoyl-1-C^{14} chloride was added. Since the amount of acid chloride added was insufficient for complete acylation of the diglycerides, there was maximum opportunity for acyl migration to occur. After 72 hours of exposure to these conditions, 1 mole of fatty acid chloride was added and the reaction allowed to proceed for an additional 72 hours to completion. The resulting triglyceride was isolated and the location of the marker fatty acid determined in it. Other samples of the diglycerides were treated by the method which is being proposed here, where acylation takes place in one step. The analytical values obtained are given in Table 3. The amount of marker fatty acid found in the 2-position of the triglyceride was the same regardless of whether acylation was carried out in one or two steps. Thus acyl migration does not occur during the acylation step. Once the diglycerides or monoglycerides have been converted into triglycerides, there is no further possibility of acyl migration.

TABLE 3. DISTRIBUTION OF MARKER FATTY ACIDS IN DIGLYCERIDES FOLLOWING ACYLATION WITH FATTY ACID CHLORIDE IN ONE OR TWO STEPS

Diglyceride Acylated	Acylation Condition	Marker Fatty Acid in 2-Position of Triglyceride
		per cent
1,2-Dipalmitin	One step	6
1,2-Dipalmltin	Two steps	7
1,3-Dipalmitin	One step	95
1,3-Dipalmitin	Two steps	95

RESULTS

Application of the Method to Monoglycerides. The results obtained on applying this procedure to monoglycerides[4] are given in Table 4. In this instance linoleic acid was used as the marker fatty acid, since none of it was present in the monoglycerides studied. For comparative purposes, the values obtained by the periodic acid method (11) are given. It will be noted that the values obtained by the two methods are in good agreement. The lipase method will probably have only limited application to monoglycerides since the periodic acid method is much easier to carry out. How-

[3] The 1,2-dipalmitin was obtained by the incomplete acylation of 2-monopalmitin, prepared by the method of Martin (7), and isolation of the diglyceride by chromatographing on silica gel (6). The 1,3-dipalmitin was prepared by the method of Baur and Lange (8).

[4] The 1-monoglyceride was synthesized from isopropylideneglycerol (10) and the 2-monoglyceride was prepared by the method of Martin (7).

ever, the values in this table have been reported for the purpose of allowing a comparison of the new method with one that is already established.

TABLE 4. STRUCTURE OF MONOGLYCERIDES AS DETERMINED BY THE PERIODIC ACID AND LIPASE METHODS

Monoglyceride Source	Per Cent 1-Monoglyceride as Determined by:	
	Periodic Acid Method	Lipase Method
2-Monopalmitin	2	3
2-Monopalmitin	10	7
1-Monopalmitin	98	98
Mixture of 1- and 2-monoglycerides	88	86

Application of the Method to Diglycerides. The structures of three different samples of 1,2- and 1,3-diglycerides determined by this procedure are given in Table 5. The marker fatty acid used in these instances was oleate-1-C^{14}. Unfortunately, there is no other method reported for determining the structure of diglycerides to which these values could be compared. Because of the ease with which one isomer of a diglyceride can be converted to the other, it is difficult to retain a diglyceride in a single isomeric form. Hence, the fact that an old sample of 1,2-dipalmitin was found to consist of 49% of the 1,3 form, and none of the other diglycerides were entirely of their supposed structure, does not mean that the method of analysis is in error by these amounts. The critical test of the procedure is the results obtained on the mixtures of 1,2- and 1,3-diglycerides. On the basis of the weight of each diglyceride used in making up the mixture and the values obtained on the individual components, the theoretical values for the 1,3-diglyceride content was calculated. The values obtained were within 5% of those expected.

DISCUSSION

It is impossible at this time to be certain of the error involved in this method. Since triglycerides of supposedly known structure are synthesized from mono- and diglycerides, their structure can be no more certain than that of the partial glycerides from which they are prepared. Thus, the ease with which mono- and diglycerides isomerize makes uncertain the absolute purity of many triglycerides whose preparation has been reported. This ready isomerization of partial glycerides also precludes the use of mono- and diglycerides as analytical standards. Hence, methods which do not rely on analytical standards must be used to determine the reliability of this method. In the case of monoglycerides, an independent method is available. A comparison of the results obtained by the two methods, as shown in Table 4, indicates the error by the lipase method to be quite small. The results obtained on the mixtures of diglycerides (Table 5) again point to a relatively small error. It is probable that the values obtained by the lipase method are within 5% of the true value. Where the original glyceride contains fatty acids less than 12 carbon atoms in chain length, the presence of esterase in the pancreatin may interfere with this method.

This method can be used also for determining the distribution of fatty acids in a diglyceride of mixed fatty acid composition. The fatty acid composition of the diglyceride is determined by one of the standard methods. Using the methods described here, the diglyceride is acylated with a marker fatty acid, digested with pancreatic lipase, and the resulting monoglycerides characterized. The marker fatty acid is used to determine the position of the free hydroxyl group in the original diglyceride. The proportion of a particular nonmarker fatty acid in the monoglycerides resulting

TABLE 5. STRUCTURE OF DIGLYCERIDES AS DETERMINED BY THE LIPASE METHOD

Diglyceride Source*	Per Cent 1,3-Diglyceride	
	Theory*	Found†
1,3-Dipalmitin		94, 96, 100
1,2-Dipalmitin		3, 5, 6
Mixture of Above 1,3- and 1,2-Dipalmitin	50	49
1,3-Dipalmitin		94, 96, 100
1,2-Dipalmitin		12, 12, 15
Mixtures of Above 1,3- and 1,2-Dipalmitin	67	67
	28	27
	46	50
1,3-Dipalmitin		92, 95, 97
1,2-Dipalmitin, old sample		49, 49
Mixtures of Above 1,3- and 1,2-Dipalmitin	71	73
	64	65
	88	89
	89	92

* Theoretical values for the mixtures of diglycerides are based on the proportions used in making these mixtures and the values obtained on the individual components by the lipase method.

† Each figure reported is that of a separate analysis.

from lipase digestion will be proportional to the amount in the 2-position of the original diglyceride.

A sequence of reactions similar to those reported here has been used by Tattrie (12) for determining the location of fatty acids in lecithin. The initial step he employs is the splitting of phosphorylcholine from lecithin. His subsequent steps are similar to those reported here for a diglyceride, except that he uses the composition of the fatty acids freed by lipase hydrolysis to determine the distribution of the fatty acids in the glyceride. Although he is careful to allow the hydrolysis to proceed only to the diglyceride stage, there is always the danger that some of the fatty acids esterified with the 2-position will appear as FFA. In the method described in the present paper, the enzymatic hydrolysis of the triglycerides is carried out in the presence of a sufficient excess of calcium so that the fatty acids which are freed are immediately converted to insoluble calcium soaps. Because of this, these fatty acids cannot take part in esterification reactions. Thus the monoglycerides which are present at the end of the enzymatic digestion have been formed solely by the successive hydrolysis of triglycerides yielding 1,2-diglycerides then 2-monoglycerides. If a portion of the 2-monoglycerides are in turn hydrolyzed, it will not influence the results, since only the monoglyceride fraction is analyzed. The advantage of using the monoglyceride rather than the FFA fraction is apparent.

The method for determining the structure of partial glycerides is not directly applicable to a mixture of mono- and diglycerides; one must first separate them by a suitable method. This isolation, or any other treatment of mono- and diglycerides, must be carried out under rigorously controlled conditions because of the ease with which these partial glycerides isomerize. For example, even a brief exposure to heat will bring both mono- and diglycerides to their normal equilibrium mixture (13).

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Countercurrent distribution of soybean fatty acid methyl esters biosynthetically labeled with H^3 and C^{14}

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SUMMARY

Fatty acids randomly labeled with C^{14} and with H^3 were prepared by culturing soybean plants (*a*) in the presence of $C^{14}O_2$, and (*b*) with H^3OH in the nutrient medium. After maturation of the beans, extraction of the oils, and transesterification of the glycerides with methanol, the methyl esters were readily separated by countercurrent distribution to yield fractions of pure linoleate (7 g and 469 μc of C^{14}-labeled esters), 97% linolenate, a 2:1 mixture of oleate and palmitate, 83% stearate, and concentrates of C_{20} and higher acid esters. Over-all recoveries of 90% may be expected from countercurrent distribution.

Biosynthetically labeled lipids have been prepared by the culture of soybeans (1), *Chlorella pyrenoidosa* (2), and *Phycomyces blakesleeanus* (3). Small amounts of individual fatty acids have been isolated in the last two investigations. For the production of labeled linoleic acid, the use of soybeans has an advantage in the large amount of oil produced, in its relatively simple fatty acid system, and in the preponderance of linoleic acid (> 50%) found in its glycerides.

Recently a countercurrent distribution procedure was described for the preparation of individual fatty acid methyl esters,[1] which employs hydrocarbon and acetonitrile solvents. The present paper describes the application of this procedure to the isolation of soybean fatty acid methyl esters which had been biosynthetically labeled during growth with tritium and C^{14}.[2]

MATERIALS AND METHODS

The culture of soybeans from seedlings to various stages of maturity in three Argonne-type plant growth isotope chambers (1, 4), (*a*) in the presence of $C^{14}O_2$, (*b*) with H^3OH in the nutrient solution, and (*c*) under control conditions will be described in another publication, together with observations of the efficiency of production of isotopically labeled soybean oil and the effects of radiation on plant growth and composition.[3] After harvesting the seed in various maturity groups, the beans were dried, ground, and extracted with pentane-hexane. Analyses for fatty acid compositions of these maturity groups were made by gas-liquid chromatographic (GLC) methods after formation of the methyl esters by transesterification with methanol. The C^{14}-labeled esters were then combined to yield 16 g, and the tritium-labeled esters to total 5 g. These samples served as starting materials for the preparative countercurrent distribution to be described.

Gas-Liquid Chromatography. A 5-foot column of

* Dutton, Jones, and Scholfield performed this work at the Northern Regional Research Laboratory; Chorney and Scully performed this work, in part, at the Argonne National Laboratory under the auspices of the U.S. Atomic Energy Commission.

[1] C. R. Scholfield and H. J. Dutton. Presented at the American Oil Chemists' Society meeting, New Orleans, La., April, 1959.

[2] W. Chorney, N. J. Scully, L. H. Mason, and H. J. Dutton. In preparation.

[3] See footnote 2.

Resoflex 296[4] (adipate polyester of diethylene glycol) on Celite® 545, operated at 200° with a helium flow rate of 50 ml per minute, was used to analyze the methyl esters in the Aerograph gas chromatograph. Approximately 500 theoretical plates are calculated for this column.

Radioactivity. Activity of both tritium and C^{14}-labeled esters was determined by use of liquid scintillation procedures (5). Toluene containing 4 g POP (2,5-Diphenyloxazole) and 30 mg POPOP (1,4-*Bis*-2-[5-Phenyloxazolyl] Benzene) per liter was used as the scintillation solution. Radioactivity of fatty acids issuing from the GLC equipment was determined by condensing the esters from the gas stream in the scintillation solution contained in individual vials for 1-minute periods and by assaying the vials with a Packard Automatic Tri-Carb Scintillation Spectrometer.

Countercurrent Distribution. Separation of the methyl esters was performed in a 200-tube automatic countercurrent distribution instrument. Acetonitrile (40 ml) and pentane-hexane (10 ml) were used as the solvent pair. In successive experiments the C^{14} esters and the H^3 esters were introduced into the first ten and the first four tubes of the instrument, respectively. After 200 transfers, the petroleum ether layers were collected by the withdrawal technique, four 10-ml aliquots being combined in each collection tube; distribution was continued until 640 transfers had been applied. At this stage, the slowest moving ester, methyl linolenate, had been "eluted" from the instrument. Radioactivity of every other collection tube was determined by introducing 0.1-ml aliquots into 15 ml of scintillation solution and by assaying the activity with the scintillation spectrometer. Based on this distribution curve for radioactivity, combinations of tubes were made, solvents were evaporated, compositions determined by GLC, and the preparations sealed in ampules *in vacuo*.

Oxidation of Methyl Oleate. The D Fractions were saponified in 95% ethanol with potassium hydroxide at reflux temperature for 45 minutes, acidified with hydrochloric acid, and the extracted acids oxidized as soaps at room temperature with a mixture of metaperiodate and permanganate (6). The mixture of monobasic and dibasic acids was recovered by extraction with ethyl ether. Conditions of oxidation as described (6) do not cause a shift in double-bond position.

[4] Cambridge Industries, Cambridge, Mass. Mention of commercial equipment or products does not constitute endorsement by the U.S. Department of Agriculture over those of other manufacturers.

Liquid-Liquid Partition Chromatography. Aliquots of the cleaved acids were applied to a silicic-acid column (7) for the separation of all monobasic acids as one peak, and of individual dibasic acids as separate peaks. Subsequently, fractions of the monobasic-acid peak were recombined and these acids were applied to a different silicic-acid column (8) to separate individual monobasic acids.

RESULTS AND DISCUSSION

A gas-liquid chromatogram for the tritium-labeled esters from mixed mature and immature beans is given in Figure 1. The curve for radioactivity parallels the

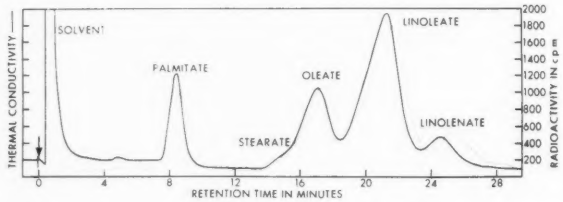

FIG. 1. Gas-liquid chromatogram of tritium-labeled methyl esters. Solid line=thermal conductivity; dotted line=radioactivity.

thermal conductivity curve, as would be anticipated if radioactive atoms were evenly distributed among the fatty acids. The radioactivity curve lags slightly behind because of the time required for the gaseous stream to flow from the conductivity detector into the scintillation solution. By integrating areas under thermal conductivity curves, the compositions of the C^{14}- and H^3-labeled samples of esters used for countercurrent distribution were calculated and are given in Table 1.

The radioactivity curve for countercurrent distribution of the C^{14}-labeled methyl esters is shown in Figure 2. Fractions A and B differed in content of C_{20} and C_{22} saturated acids, but detailed analyses of these minor fractions were not made. Fraction C, as calculated from GLC data, is 85.2% methyl stearate. Similarly, Fraction D was composed of 0.3% stearate, 32.7% palmitate, and 67.1% "oleate." (The GLC analyses would not have distinguished C_{18} monoenes with double bonds in the 8, 9, and 10 positions.) In this pentane-hexane acetonitrile system, the effect of introducing a double bond into methyl stearate, i.e., methyl oleate, is approximately equal to the effect of reducing the chain length by 2 carbons, i.e., methyl palmitate. If separation of this binary system were

TABLE 1. COMPOSITION OF H^3- AND C^{14}-LABELED METHYL ESTERS AND FRACTIONS SEPARATED BY COUNTERCURRENT DISTRIBUTION

Fractions	Transfer No.	Approximate Weight Recovered	Per Cent of Total Recovered Weight	Fatty Acid Compositions					
				Stearic	Palmitic	Oleic	Linoleic	Linolenic	C_{20} Higher
	H^3-labeled esters	*mg*		2.5	11.0	23.6	53.5	9.3	
A	200–231	37.3	1.14	1.2					98.9
B	232–263	50.5	1.54	2.1					97.9
C	264–287	154.3	4.70	83	12				5.0
D	288–366	1,054.0	32.15	0.5	32.8	66.7			
E	367–494	1,760.0	53.7				99+		
F	495–681	222.0	6.8				3.1	96.9	
		3,278.1							
	C^{14}-labeled esters			2.0	11.05	23.3	53.1	10.6	
A	201–224	70.2	.5						100
B	225–252	202	1.5	.8					99.2
C	253–288	595	4.4	85.2	9.6				5.2
D	289–368	4,250	31.7	.3	32.7	67.1			
E	369–492	7,040	52.6				99+		
F	493–640	1,230	9.2				2.1	97.9	
		13,387.2							

required, a urea crystallization or micro distillation prior to distribution would readily remove the palmitate.

The gas-liquid chromatogram for methyl linoleate of Fraction E is shown in Figure 3. Greatly overloading the column with Fraction E revealed only traces of impurities in this preparation. Because of current interest in the essential fatty acid function of linoleic acid, this ester is of particular value for biological experiments. Fortunately, as shown in Figure 3, it is isolated in high purity. Because a tail of the E fraction entered the F fraction, 2.1% of linoleate contaminated the linolenate. Comparable data for tritium-labeled soybean esters were obtained (Table 1).

In the countercurrent distributions for H^3- and C^{14}-labeled esters, 70% and 90%, respectively, of the esters introduced could be accounted for. Furthermore, the percentages of the recovered weight in the individual fractions closely approximate the composition of the starting materials and are indicative of no selective losses during countercurrent distribution. This isola-

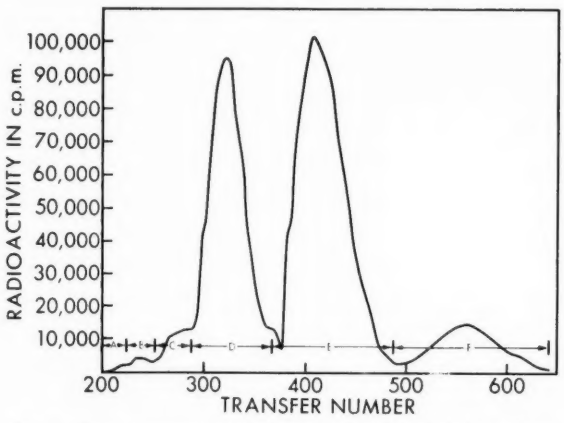

FIG. 2. Countercurrent distribution of C^{14}-labeled soybean methyl esters.

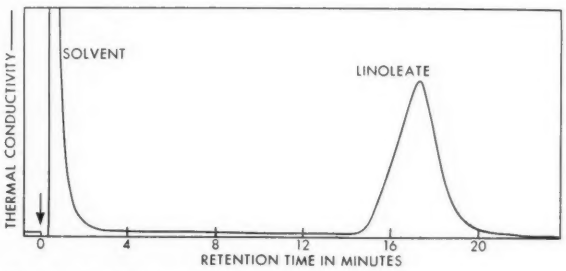

FIG. 3. Gas-liquid chromatogram of Fraction E.

tion of methyl esters can be readily carried out by laboratories possessing the requisite equipment. The extraction of ground beans, and countercurrent distribution operations described, were carried out by two people working over a period of two weeks. The amounts isolated are indeed practical for conducting subsequent experiments.

Although the specific activities of these preparations are low for these fatty acid esters, 0.067 μc per mg for C^{14} and 0.026 μc per mg for H^3, preliminary observations[5] indicate that at least a tenfold increase in specific activity might be expected by alteration of cultural methods and labeling; in addition, the total yield of seed might be increased manyfold.

Since the soybean plants were grown from seedling stage to maturity in the presence of isotopes, random labeling of constituents would seem to be obligatory. In agreement with this inference, no selectivity for a given isotope between individual fatty acids is evident from data such as given in Figure 1 in which the ratio of radioactivity to thermal conductivity current appears to be constant for all fatty acids.

Random labeling among the carbon atoms of the fatty acid is also indicated by comparing the activities of palmitic acid and of the oxidative cleavage products of oleic acid in Table 2. Thus the activities per carbon atom for palmitic acid (1225), azelaic acid (1160), and pelargonic acid (1276) after the periodate-permanganate oxidation of C^{14}-labeled Fraction D are equal within limits of error. In Figure 4 a liquid-liquid partition chromatogram for oxidative scission acids is given for a column designed to separate dibasic from monobasic acids. The curve for dibasic acids was obtained by collecting, titrating, and counting alternate 2-ml

TABLE 2. RADIOACTIVITY (CPM/1000 PER MILLIMOLE) OF OXIDATIVE CLEAVAGE PRODUCTS FROM H^3- AND C^{14}-LABELED ESTERS OF FRACTION D

Trial No.	Dibasic Chromatograms				Monobasic Chromatogram of Monobasic Mixture*		
	Monobasic*	Dibasic		Recovery	C_{16}	C_9	Recovery
		C_{10}	C_9				
				per cent			*per cent*
			H^3 Fraction				
1	1388	1460	1016	97.8	2052	1433	93.4
2	1712	—	1054	108.4	1992	1313	90.5
Av.	1550	1460	1035	103.1	2022	1373	92.0
Activity per H atom		91	74		65	81	
			C^{14} Fraction				
1	13380	13850	10260	89.1	19860	11650	90.2
2	15260	15050	10630	93.4	19380	11350	88.4
Av.	14320	14450	10445	91.3	19620	11500	89.3
Activity per C atom		1445	1160		1225	1276	

* This includes the palmitic acid present before oxidation as well as those monobasic acids produced upon oxidation.

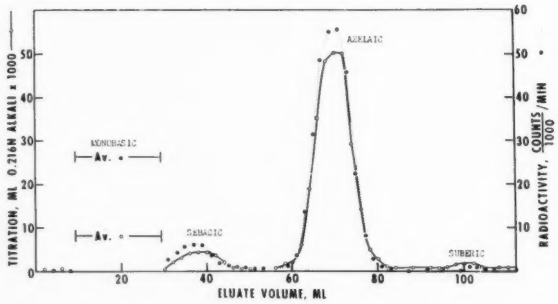

FIG. 4. Liquid-liquid partition chromatogram of dibasic acids from the periodate-permanganate oxidized Fraction D. Titration and activity of the mixed monobasic acid group are indicated as average values. (See Fig. 5.)

[5] Unpublished data from the Argonne National Laboratory.

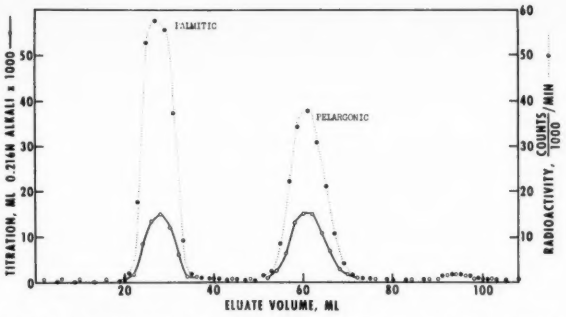

FIG. 5. Liquid-liquid partition chromatogram of the mixed monobasic acid group shown in Figure 4.

portions of eluate. The monobasic fraction was collected in one tube and aliquots were titrated and counted to give the average values of Figure 4. The rest was concentrated, reassayed for radioactivity, and applied to a second column designed to separate monobasic acids (Fig. 5). Alternate fractions in each of the chromatograms were assayed for radioactivity by liquid scintillation methods, and were titrated with 0.216 N NaOH using a Gilmont microburette. The data for C^{14}-labeled esters given in Figures 4 and 5, and from a duplicate oxidation and chromatograms (not shown), are presented in Table 2. Similar data of H^3-labeled esters were obtained and are also summarized in Table 2. The presence of sebacic and suberic acids (Fig. 4) is not unexpected. Jones and Stolp (6) have shown that these acids may be found when "pure" oleic acid is cleaved by the same procedure.

The constancy of C^{14} activity per carbon atom for these scission acids is indicative of random labeling in the intact fatty acids. The slight deviation from constancy of H^3 activity per hydrogen atom for oleic acid cleavage products may be explained in part by the exchange of tritium atoms in labile positions during saponification and oxidation cleavage reactions.

The authors are indebted to Benita Goett for her assistance in various phases of the conduct of this work, and to J. C. Cowan for his interest and encouragement.

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Naturally occurring epoxy acids: I. detection and evaluation of epoxy fatty acids by paper, thin-layer, and gas-liquid chromatography*†

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SUMMARY

Chromatographic procedures for the detection and evaluation of oxygenated fatty acids are described. Emphasis has been placed on epoxy acids, but these methods promise to be of great value in studies of all classes of oxygenated acids. Paper chromatography of fatty acids and their esters has been developed for the examination of mixtures containing oxygenated derivatives. The method of adsorption chromatography on thin layers of silicic acid has been shown to be a powerful tool in studies of epoxy acids and hydroxy acids. Gas-liquid chromatography (GLC) of epoxy esters has been studied using both polar and nonpolar columns and has great utility in the detection and analysis of these compounds in mixtures. These methods have been applied to the examination of the epoxy components of six seed oils. Thin-layer and GLC proved particularly useful in this study and together demonstrated the presence of at least three distinct epoxy acids in each of these oils. Some conclusions as to the probable structures of these epoxy components are presented on the basis of their chromatographic characteristics in relation to known substances.

The first epoxy acid shown to occur naturally, *cis*-12:13-epoxyoctadec-9-enoic acid,[1] was discovered and characterized by Gunstone (1) in 1954. It comprised 72% of the mixed acids of *Vernonia anthelmintica* seed oil. Since then the same acid has proved to be a constituent of several other oils (2, 3, 4) and its isomer, *cis*-9:10-epoxyoctadec-12-enoic acid has been described as a component of *Chrysanthemum coronarium* seed oil (5). These two acids are obviously structurally related to linoleic acid. Epoxy acids similar structurally to linolenic acid and oleic acid have also been characterized. An example of the former, 15:16-epoxyoctadec-9,12-dienoic acid, occurs in *Camelina sativa* seed oil (6), and the latter, cis-9:10-epoxystearic acid comprises 28% of the mixed acids from the uredospores of a wheat stem rust (7) and 3% of the acids of *Tragopogon porrifolius* seed oil (8). Many other seed oils have now been shown to have epoxy components (9, 10, 11), not yet characterized, and a widespread distribution of these acids in nature is now becoming recognized.

The above-mentioned structural relationship between the epoxy acids thus far described and the common unsaturated acids poses the question of a more general relationship between the two classes of compounds. The possibility exists that the epoxy group is a precursor of unsaturation or a product of the metabolism of unsaturated acids. These compounds may therefore be of greater importance in lipid chemistry than previously imagined, and improved methods for their detection and evaluation in small amounts are desirable.

Most methods in general use for the detection and estimation of long-chain epoxy compounds depend on the uptake of hydrogen halide by the epoxy group. Measurement of hydrogen halide uptake can be done directly (12), potentiometrically (13), or by back

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[1] The epoxy ring structure is indicated by a colon, e.g., 12:13-epoxyoctadec-9-enoic acid, which is synonymous with 12,13-epoxyoctadec-9-enoic acid.

titration (14, 15, 16), and unless fairly large samples are available little reliance may be placed on results indicating much below 5% of epoxy component in a fat. In some cases much higher apparent epoxy values may be completely spurious (10, 11, 15). Of other methods which have been tried, polarography offers little promise (17) and colorimetry (18) has not yet been developed for long-chain epoxides. Proton magnetic resonance (19) is useful only with concentrations higher than 5%, and glycol determination by periodate oxidation, after splitting the epoxide group (20), is more tedious and less sensitive than the standard methods.

In view of the possible importance of epoxy compounds in lipid chemistry, more sensitive methods are required for their detection and measurement. This paper describes the development of paper, thin-layer, and gas-liquid chromatographic methods for this purpose.

EXPERIMENTAL

Materials. *cis*-12:13-Epoxyoleic acid was prepared from *Vernonia anthelmintica* mixed acids by partition between 80% methanol and hexane (20). A portion of this acid, which had oxirane oxygen 5.2% (theory 5.4%), was esterified by reaction with ethereal diazomethane.

cis-9:10-Epoxystearic acid (m.p. 57.5°-58°; reported 59.5°), *trans*-9:10-epoxystearic acid (m.p. 53°-54°; reported 55.5°), and *cis,cis*-9:10,12:13-diepoxystearic acid (m.p. 78°-78.5°; reported 79°) were prepared by the methods of Swern *et al.* (21, 22). The methyl esters were prepared by reaction with diazomethane in anhydrous ether solution. *threo*-12,13-Dihydroxyoleic acid (m.p. 50°-52°; reported 53°-54°) was derived from methyl epoxyoleate by reaction with acetic acid followed by hydrolysis (1) and the ester prepared with diazomethane. The mixed isomeric 12,-13-chlorohydroxyoleic and 13,12-chlorohydroxyoleic acids or esters were prepared from 12:13-epoxyoleic acid or ester by the action of anhydrous ethereal hydrogen chloride (14). 12-Hydroxyoleic acid (ricinoleic acid) was isolated from the mixed acids of castor oil by partition between 80% methanol and hexane (20). A portion of the acid was esterified with methanolic hydrogen chloride.

The esters of the oils which were studied by the methods described here were obtained by alkaline hydrolysis at room temperature, acidification, and esterification of the resultant acids with diazomethane. Prolonged contact of samples with mineral acids during acidification was avoided.

Paper Chromatography. Whatman No. 1 paper was siliconized by immersion in a solution of 4% of Dow Silicone No. 200[2] in ethyl ether. The procedures used have been described by Schlenk *et al.* (23, 24). Chromatograms were stained with iodine vapor to develop unsaturated components, or α-cyclodextrin and iodine for saturated components. The common solvent systems used in the study of fatty derivatives are aqueous acetic acid or aqueous peracetic acid mixtures. These are unsuitable for studies of oxygenated acids and esters because, with the usual proportions of acetic acid, epoxy compounds react and migrate to the solvent front along with any hydroxy components. Aqueous acetonitrile and aqueous tetrahydrofuran systems, however, have been found to be suitable for studies of these compounds.

Free acids tend to smear during chromatography with the acetonitrile systems, due to dimerization. This has been prevented by the addition of 2% acetic acid to the solvent, producing discrete spots. However, the tetrahydrofuran system used gave little smearing with free acids.

R_f values of standard acids and methyl esters, using aqueous acetonitrile and aqueous tetrahydrofuran systems, are listed in Table 1.

The acetonitrile systems are shown to be most suitable for studies of oxygenated derivatives since non-oxygenated saturated and unsaturated acids or esters migrate very little and can be readily distinguished from epoxy and hydroxy components. A mixture of monoepoxy, monohydroxy, diepoxy, and dihydroxy C_{18} acids or esters can be separated into the four components. Such a separation is not possible with the aqueous tetrahydrofuran systems where diepoxy and monohydroxy derivatives have similar R_f values. A consideration of time also favors the acetonitrile systems since a solvent front migration of 25 cm takes only 5 to 6 hours, whereas the tetrahydrofuran systems require more than 24 hours for the same migration.

The tetrahydrofuran systems give less separation between classes of compounds, so that careful measurement and comparison with standards is necessary in studies of unknown mixtures. However, this system may be useful as a confirmation of the results of the aqueous acetonitrile system.

Paper chromatography will detect less than 1% of epoxy component in a mixture and, if esters are prepared with radioactive diazomethane, the components

[2] Dow Corning Corporation, Chicago 1, Ill.

TABLE 1. R_f VALUES OF FATTY ACIDS AND THEIR METHYL ESTERS IN PAPER CHROMATOGRAPHY*

Compound	Esters		Acids	
	MeCN/H_2O 50/50	C_4H_8O/H_2O 75/25	MeCN/H_2O/AcOH 45/53/2	C_4H_8O/H_2O 80/20
$C_{16:1}$ + $C_{18:1}$	0.05 + 0.02	0.46 + 0.42	0.10 + 0.04	0.70 + 0.63
9:10-epoxy $C_{18:0}$	0.28	0.51	0.48	0.74
12:13-epoxy $C_{18:1}$	0.33	0.55	0.59	0.78
9:10,12:13-diepoxy $C_{18:0}$	0.61	0.67	0.84	0.95
12-hydroxy $C_{18:1}$	0.50	0.65	0.72	0.88
12,13-dihydroxy $C_{18:1}$	0.80	0.92	0.91	0.99
12,13-chlorohydroxy $C_{18:1}$ and 13,12-chlorohydroxy $C_{18:1}$	0.48	0.58	0.74	0.83

MeCN = acetonitrile; C_4H_8O = tetrahydrofuran; AcOH = acetic acid.
* Samples were approximately 5λ of 1% solutions, i.e., about 0.05 mg.

can be estimated quantitatively by the method of Mangold *et al.* (25).

Thin-layer Chromatography. The separation of mixtures by adsorption chromatography on silica gel, in the form of a thin layer on a glass plate, has been developed by Stahl (26, 27). This very elegant method for the separation of different classes of compounds has been applied to the analysis of lipid materials by Mangold and Malins (28, 29). Details of the preparation of chromatoplates and of their use may be found in these references.

After elution of the chromatogram, the position of components can be determined by the methods of paper chromatography (24, 25). A more general method of detection, however, is applicable to thin-layer chromatograms and was used for most of this work. This consists of spraying the plate with 50% sulfuric acid and heating until all organic constituents are charred and appear as black spots. Moreover, the rate of appearance and the initial color of individual spots give qualitative information regarding the degree and type of unsaturation of the components. Thus conjugated triene or α-hydroxydiene components appear as brown spots immediately after spraying, and conjugated dienes produce brown spots shortly after heating. Nonconjugated unsaturated components appear as yellow spots after a longer heating period and have changed color to brown before yellow spots, due to saturated components, appear. Spots can also be made visible by their absorption or fluorescence in ultraviolet light after spraying the plate with a 0.2% solution of 2′,7′-dichlorofluorescein in ethanol.

When thin-layer chromatography was applied to the examination of long-chain esters, it was found that, by varying the polarity of the solvent, one class at a time could be made to migrate. For instance, 1% ethyl ether in petroleum ether (b.p. 35°-45°) caused only the common saturated and unsaturated esters to migrate, as a class, at an R_f value of about 0.4, all other components remaining at the starting point. The saturated and unsaturated esters migrated close to the solvent front when the proportion of ethyl ether was increased to 5%, and monoepoxy esters then moved at an R_f value of about 0.3. If 10% ethyl ether was used (Fig. 1), monoepoxy esters migrated at about R_f 0.7, monohydroxy esters moved at R_f 0.2, diepoxy stearate moved at R_f 0.1, and dihydroxy derivatives remained on the base line. Petroleum ether containing 20% ether caused the migration of these constituents at R_f values of 1.0, 0.5, 0.3, and 0.1, respectively.

With acids it was found to be impossible to move only one class at a time, as with esters. This is because the high polarity of the carboxyl groups of acids reduces the differences between classes. However, each class has a different R_f value and was separated from the others. Figure 1 demonstrates the migration of various fatty acids with a 10% ethyl ether-petroleum ether solvent containing 1% acetic acid to prevent smearing. The nonoxygenated acids migrated close to the solvent front, with monoepoxy acids near R_f 0.6 and diepoxystearic acid at 0.1. Monohydroxyoleic acid (ricinoleic acid) moved to an R_f value of about 0.3, and dihydroxyoleic acid remained at the starting point.

The chlorohydrins from 12:13-epoxyoleic acid and

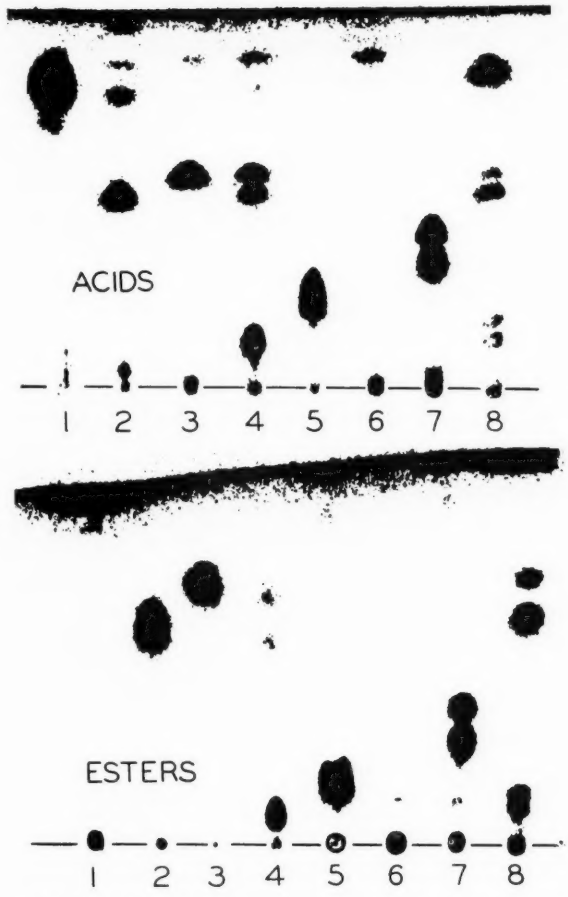

FIG. 1. Thin-layer silicic-acid chromatography of known compounds. Solvent used for esters was 10% ethyl ether in petroleum ether (b.p. 35°-45°) and for acids was the same with added 1% of acetic acid. Spots were developed by heat, after spraying with 50% sulfuric acid, and reproduced by photocopying. 1. Palmitoleic and oleic; 2. *cis*-9:10-epoxystearic; 3. *cis*-12:13-epoxyoleic; 4. *cis,cis*-9:10,12:13-diepoxystearic plus two monoepoxy-impurities; 5. 12-hydroxyoleic; 6. *threo*-12,13-dihydroxyoleic; 7. *threo*-12,13-chlorohydroxyoleic and *threo*-13,12-chlorohydroxyoleic; 8. *Artemisia absinthium* mixed acids and mixed esters.

ester were shown to be intermediate in migrating speed between the corresponding epoxy and hydroxy derivatives. This is probably caused by intramolecular hydrogen bonding between the hydroxy and chloro groups, reducing the polarity of the hydroxy group. The two possible isomers were clearly separated on both plates (Fig. 1.) Considerations of structure in relation to hydrogen bonding suggest that the 13-chloro-12-hydroxyoleic derivative may have the higher R_f value.

It is also noteworthy that the two mono-epoxidized linoleic acids and esters, present as impurities in the diepoxystearic samples, were clearly separated. The upper spot is identical with that of the 12:13-epoxyoleic derivative and the lower spot therefore represents the 9:10-epoxyoctadec-12-enoic compound.

It should be noted here that the 9:10-epoxyoctadec-12-enoic acid and ester have the same R_f values, respectively, as 9:10-epoxystearic acid and ester. These compounds are inseparable by this method but can be distinguished by other methods (*vide infra*).

This ability to separate very similar compounds makes thin-layer chromatography a more useful method, in many cases, than paper chromatography, which does not separate the two pairs of isomeric compounds described above. As a further example of this difference in resolving power, the esters from *Artemisia absinthium* seed oil, when separated by paper chromatography (30), showed one spot corresponding to an epoxy ester and one corresponding to a monohydroxy ester. Thin-layer chromatography, however, demonstrated the presence of two epoxy esters, two monohydroxy esters, and at least one dihydroxy derivative (Fig. 1). The sensitivity of thin-layer chromatography is such that about 0.1% of an epoxy component can be detected in a 1 mg sample of mixed acids or esters, as shown with *Dimorphotheca aurantiaca* esters in Figure 3. This oil contains approximately 0.6% of epoxy acids (11) yet three epoxy components are clearly visible. Radioactive esters, prepared with carbon-labeled diazomethane, can be separated by thin-layer chromatography and a quantitative estimation of epoxy and other oxygenated components obtained by the method of Mangold (31).

Gas-liquid Chromatography. Three liquid phases were used to evaluate this method for studies of epoxy compounds: The nonpolar Apiezon L grease,[3] and the polar LAC-2-R446[4] and Craig[5] polyesters, coated on acid and alkali washed Celite® in copper tubing. Further details are given under Figure 3 and Table 3. Detection was by β-ray ionization using either Research Specialties Company Model 600-2 or Barber-Coleman Model 10. Samples were injected as 1% solutions in methyl hexanoate or acetone in 5 to 10λ amounts.

[3] Registered: Metropolitan-Vickers Electrical Co., Ltd., England.

[4] Diethylene glycol adipate and pentaerythritol adipate polyesters, obtainable from Cambridge Instrument Co., Cambridge, Mass.

[5] Butanediol-succinate polyester (32).

Mixtures of straight-chain saturated fatty esters with pure samples of methyl *cis*-12:13-epoxyoleate, *cis*-9:10-epoxystearate and *trans*-9:10-epoxystearate were injected to determine the retention characteristics of each column. The results are presented as semilogarithmic graphs in Figure 2.

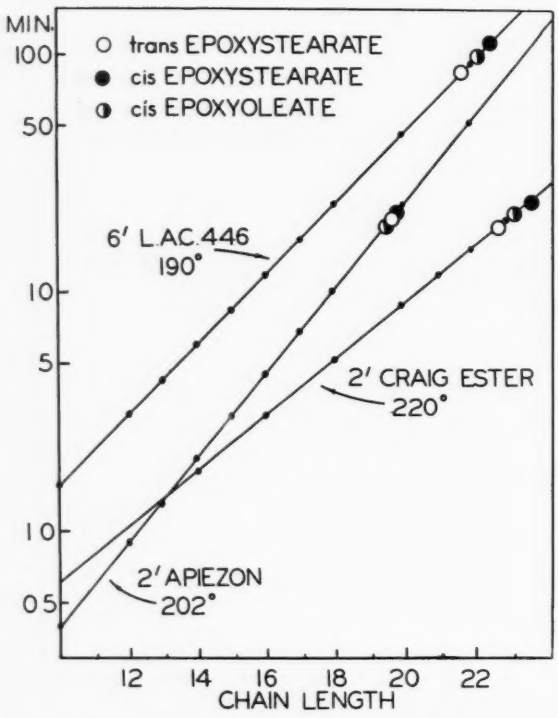

FIG. 2. The relation between the logarithm of the retention time and the chain length for some normal saturated acid methyl esters and methyl *cis*-12:13-epoxyoleate; methyl *cis*-9:10-epoxystearate and methyl *trans*-9:10-epoxystearate. Conditions: 1. LAC-2-R446, 16 g (16.5%) on 80 to 100 mesh acid and alkali-washed Celite®. Column 6′ × ¼″ at 190° and flow rate 62 ml/minute. 2. Craig polyester, 6 g (20%) on 80 to 100 mesh acid and alkali-washed Celite®. Column 2′ × ¼″ at 220° and flow rate 67 ml/minute. 3. Apiezon L, 5 g (17%) on 60 to 100 mesh acid and alkali-washed Celite®. Column 2′ × ¼″ at 202° and flow rate 71 ml/minute. Flow rates calculated at S.T.P.

On the polyester columns these epoxy esters emerged with "carbon numbers" of 22-23 and 23-24, respectively, and the order of emergence was *trans*-epoxystearate, *cis*-epoxyoleate and *cis*-epoxystearate. On the nonpolar column, however, they had a lower retention time and emerged with "carbon numbers" 19-20, *cis*-epoxyoleate having the shortest retention time followed by *trans*- and *cis*-epoxystearates. None of these columns separated the individual epoxy esters completely and mixtures appeared as composite peaks. However, a longer nonpolar column, 4-foot, Apiezon L, operated under similar conditions, clearly separated *cis*-epoxyoleate from *cis*-epoxystearate (*vide infra*) and longer polyester columns may similarly improve separation.

We have recently described the alteration, during gas-liquid chromatography (GLC), of several hydroxy and other acids (33) and the possibility of alteration of epoxy esters was not overlooked. No change in composition of epoxy esters was noted. Epoxyoleate, for example, was collected after GLC and chromatographed alongside the original ester on a thin-layer plate. Both samples had the same R_f value.

The sensitivity of detection of methyl epoxyoleate in admixture with C_{16} and C_{18} esters was found to be of the order of 0.1% if sufficient sample was injected.

Quantitative results were obtained by the use of added epoxy ester as an internal standard. Areas under curves were determined by cutting out peaks and weighing the paper. The results obtained using this method to determine the epoxyoleate content of some standard mixtures and of the mixed esters from *Vernonia anthelmintica* seed oil are shown in Table 2. The epoxyoleate values obtained agree well with the known values.

The method of internal normalization of the areas under curves was not suitable for mixtures containing epoxy esters. The β-ionization detector, under the conditions used, was found to give a lower response with epoxy esters than with unoxygenated esters.

TABLE 2. QUANTITATIVE ESTIMATION OF EPOXYOLEATE BY GAS-LIQUID CHROMATOGRAPHY

Sample*	Epoxyoleate	
	Actual	Estimated
	per cent	*per cent*
Standard mixture 1	2.0†	1.95
Standard mixture 2	5.0†	5.0
Standard mixture 3	10.0†	9.7
Vernonia mixed esters	71.9‡	73.0

* The standard mixtures 1, 2, and 3 consisted of methyl epoxyoleate in the amounts shown, made up with a mixture of methyl oleate and methyl palmitate (1:9 by weight).

† By weight.

‡ By chemical analysis (1).

Applications. The methods described above were applied in a study of the epoxy components of the seed oils of *Dimorphotheca aurantiaca, Artemisia absinthium, Calliandra eriophylla, Balanites aegyptica, Cosmos bipinnatus,* and *Helianthus annuus.* All these oils give epoxy values by the Durbetaki and Swern methods which are higher than the true epoxy content (30). We have shown them to contain isomers of dimorphecolic acid (9-hydroxy-*trans*-*trans*-10,12-octadecadienoic acid) and these acids are responsible for the errors in conventional epoxide determinations (10). However, we have shown that all these oils contain epoxy constituents (11) and their presence is confirmed by the methods described in this paper.

Paper chromatography of the esters derived from these seed oils (see Reference 30 for illustration) showed the presence of an epoxy constituent in every sample except that from *Dimorphotheca* oil. Thin-layer chromatography of the acids or esters from these oils presented a more complete and more informative picture of their epoxy constituents. A chromatogram of the esters from the oils run alongside known epoxy esters is shown in Figure 3.

Artemisia and *Calliandra* esters both show two well-defined spots produced by epoxy constituents. These correspond, in R_f values, to the two mono-epoxidized linoleate isomers which were present as impurities in the standard sample of diepoxystearate. The upper spot in this standard is shown to be the 12:13-epoxyoctadec-9-enoate isomer by comparison with the authentic sample of that ester, and the lower spot then is 9:10-epoxyoctadec-12-enoate. The major epoxy constituent of *Artemisia, Calliandra, Cosmos,* and *Helianthus* corresponds to this 9:10-epoxy ester and all demonstrate an upper spot corresponding to the 12:13-epoxyoleate. *Balanites* esters show their main epoxy constituent to be the 12:13-epoxyoleate with a small trace of the more polar isomer. The *Dimorphotheca* sample, however, besides showing both these components, has its main epoxy constituent at an even higher R_f value.

The R_f values of 9:10-epoxystearate, 12:13-epoxyoleate and this last compound increase linearly (0.43, 0.51, and 0.59). This suggests that it could be 15:16-epoxylinoleate, which has already been identified as a constituent of the seed oil of *Camelina sativa* (6). However, the spot on the thin-layer chromatogram of *Dimorphotheca* esters representing this component appeared as quickly after spraying with H_2SO_4, as that produced by the diene-hydroxy constituent, indicating the presence of a system of conjugated double bonds. That this spot of the thin-layer chromatogram of *Dimorphotheca* esters represents two epoxy components, one of which is novel, is indicated by GLC and other studies recorded below.

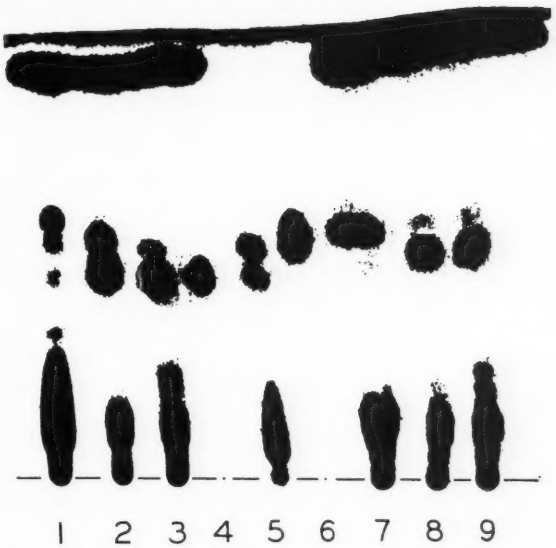

FIG. 3. Thin-layer silicic-acid chromatography of mixed esters from seed oils and standard epoxy esters. Solvent was 5% ethyl ether in petroleum ether (b.p. 35°-45°). Spots were developed by heat, after spraying with 50% sulfuric acid, and reproduced by photocopying. 1. *Dimorphotheca;* 2. *Artemisia;* 3. *Calliandra;* 4. *cis*-9:10-epoxystearate; 5. *cis,cis*-9:10,12:13-diepoxystearate and monoepoxy isomers; 6. *cis*-12:13-epoxyoleate; 7. *Balanites;* 8. *Cosmos;* 9. *Helianthus.*

It is obvious from Figures 1 and 3 that 9:10-epoxystearate and 9:10-epoxyoctadec-12-enoate have very similar migration characteristics on thin-layer chromatography. Consequently, it cannot be decided from R_f values alone whether the natural epoxy esters with the same R_f values as these two compounds are saturated or unsaturated. The observation that sulfuric acid develops color faster with more unsaturated constituents was used to determine if the natural epoxy components were saturated or unsaturated. It was found that as heating progressed, the spots produced by 12:13-epoxyoleate, its 9:10-epoxy isomer and the corresponding natural epoxy esters all appeared some time before the spots produced by mono- and diepoxystearates became visible. The conclusion that the natural epoxy esters from the oils were unsaturated was confirmed by developing a chromatoplate with iodine vapors, after spraying with a 1% solution of α-cyclodextrin. Only the spots produced by epoxystearate and diepoxystearate appeared white; all others became dark, showing unsaturation. These results prove that the lower of the two "epoxyoleate" spots, in each case,

contains an unsaturated epoxy ester, probably 9:10-epoxyoctadec-12-enoate. They do not, however, exclude the possibility that 9:10-epoxystearate may also be present in each sample as another component of this spot. GLC studies resolved this question (*vide infra*) and showed that both saturated and unsaturated components were represented in the lower spot from each of these oils.

Although not obvious from the reproduction used as Figure 3, the original chromatoplates demonstrated trace components in all samples corresponding to the "epoxylinoleate" spot of *Dimorphotheca*. A trace component with an even higher R_f value than this ester was present in all samples but no conclusions as to its structure have been reached.

Gas chromatographic analysis of the separate epoxy components from each sample was carried out on a nonpolar column. Epoxy constituents were first separated from the mixed ester samples by thin-layer chromatography using 10% ethyl ether in light petroleum as solvent. The spots produced by individual epoxy components were made visible under ultraviolet light by spraying with dichlorofluorescein, were scraped from the plate, and eluted from the adsorbant with ether (29). By this method individual components were isolated in amounts up to several milligrams. The resulting solutions were concentrated, analyzed by GLC, and the results are given in Table 3. The GLC column used here was unable to separate 9:10-epoxyoctadec-12-enoate from 12:13-epoxyoctadec-9-enoate but readily separated 9:10-epoxystearate from these isomers. Table 3 shows that all six oils contain two monounsaturated epoxy isomers, probably the two mentioned above, and a saturated epoxy acid. In addition, the components from the main epoxy ester spot from *Dimorphotheca* oil, on GLC, gave a minor peak with the carbon number to be expected for 15:16-epoxylinoleate and a pronounced peak emerging later

TABLE 3. CARBON NUMBERS* (GAS-LIQUID CHROMATOGRAPHY)† OF EPOXY COMPONENTS ISOLATED FROM THIN-LAYER CHROMATOGRAMS

Oil	Epoxy Ester Spot‡	9:10 ep. $C_{18:0}$	9:10 ep. $C_{18:1}$	12:13 ep. $C_{18:1}$	15:16 ep. $C_{18:2}$	Other	Ratio§
Dimorphotheca	1	19.8	19.5				1:3
	2			19.4			
	3				19.1	20.0	1:17
Artemisia	1	19.8	19.5				1:12.5
	2			19.4			
Calliandra	1	19.7	19.4				1:12
	2	19.7		19.4			1:7
Balanites	1	19.9	19.5				1:23
	2			19.4			
Cosmos	1	19.9	19.5				1:0.9
	2			19.4			
Helianthus	1	19.8	19.4				1:7
	2			19.4			
Standards:							
ep. $C_{18:0}$		19.9					
12:13 ep. $C_{18:1}$				19.4			
ep. $C_{18:1}$ + ep. $C_{18:0}$		19.8		19.4			
impure diep. $C_{18:0}$			19.4			21.1	

* Methyl palmitate, under these conditions, had a retention time of 16 minutes.

† Column was 4′ × ¼″ packed with 60 to 100 mesh celite (acid and alkali washed) coated with Apiezon L grease (11 g, 17%). Column temperature was 200° and flow rate 54 ml/minute (S.T.P.).

‡ Isolated from thin-layer chromatograms. Numbering of epoxy ester is from the bottom up (cf. Fig. 3).

§ Ratio of GLC peak areas of earlier to later emerging components.

than epoxystearate. The corresponding trace components of the other five oils were not examined but may be similar.

Although structural studies by degradation have not been carried out, we consider that all of these oils contain *cis*-9:10-epoxystearic acid, *cis*-9:10-epoxyoctadec-12-enoic acid, and *cis*-12:13-epoxyoctadec-9-enoic acid in varying proportions. In addition, *Dimorphotheca* oil, and possibly each of the others, contains a trace of 15:16-epoxyoctadec-9,12-dienoic acid and a novel epoxy acid of unknown constitution. This last epoxy acid constitutes about 0.3% of the acids of *Dimorphotheca* oil. Its ester, isolated by thin-layer chromatography, showed a conjugated diene absorption band at 231 mμ in the ultraviolet spectrum, a *trans,trans* conjugated diene band at 10.07 μ and possible epoxy bands at 11.44 and 11.90 μ in the infrared spectrum. Reaction with ethereal HCl altered the ester, the single product appearing on a thin-layer chromatogram lower than the pair of chlorohydrins from epoxyoleate. Boiling with acetic acid, followed by hydrolysis and esterificathe position of a dihydroxy acid. These results are all tion also altered the ester and resulted in a spot in explicable if an epoxy-conjugated-diene structure is present. Assuming some relationship with the main component acid, 9-hydroxy-*trans,trans*-10,12-octadecadienoic acid (34, 10), it seems possible that this unknown acid of *Dimorphotheca* oil is 8:9-epoxyoctadec-*trans-trans*-10,12-dienoic acid.

CONCLUSIONS

Reversed-phase partition chromatography on paper, thin-layer adsorption chromatography on silicic acid, and GLC are methods eminently suitable for the study and analysis of fatty materials containing oxygenated acids. Although emphasis has been placed on studies of epoxy compounds, the chromatographic properties of certain hydroxy compounds on paper and thin-layer plates have been described and these methods are as useful for hydroxy acids as for epoxy acids.

Thin-layer chromatography is preferable to the older techniques of paper chromatography for studies of these oxygenated derivatives. It is far more sensitive, requires less material, and readily resolves pairs of compounds which are inseparable on paper. The potential of thin-layer chromatography is amply demonstrated in the studies of seed oils recorded here and elsewhere (30). The possibility of isolating individual components from a thin-layer chromatogram in milligram amounts and carrying out gas chromatographic, ultraviolet, and infrared spectral studies on these components makes this technique doubly important.

GLC is also a powerful tool for the detection and estimation of epoxy components in seed oils. We have shown that the use of this technique in conjunction with thin-layer chromatography gives a very powerful method for studies of epoxy esters. Thin-layer chromatography, for example, will readily separate 12:13-epoxyoleate and its 9:10-epoxy isomer but will not separate the latter from 9:10-epoxystearate. GLC, on the other hand, will not separate these unsaturated epoxy isomers but will readily separate (on Apiezon L) either of these from epoxystearate. Use of both methods therefore enables all three components to be detected in a mixture.

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Naturally occurring epoxy acids: II. detection and measurement of long-chain epoxy acids by near infrared spectrophotometry*†

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SUMMARY

A new method for the detection and estimation of long-chain epoxy acids in seed oils is described. It depends on the measurement of increased absorption at 2.795 μ in the near infrared spectrum caused by chlorohydrins produced from epoxides by treatment with anhydrous ethereal hydrogen chloride. The method is sensitive to approximately 0.2% of epoxy acid in an oil and is specific for epoxides. Hydroxy components of a sample do not interfere since the strongly associated hydroxyl band of chlorohydrins is normally clearly resolved from other OH absorption. The presence of large amounts of vicinally unsaturated hydroxy acids, however, results in large changes in absorption intensity in the 2.8 μ region on HCl treatment and in these cases epoxide concentration cannot be accurately measured but must be estimated. These reactive hydroxy acids, which lead to spurious epoxide values by the conventional methods, lose hydroxyl during the acid treatment, and measurement of the decrease in their absorption at 2.762 μ means that their concentration may be estimated concurrently with that of epoxy components. Other reactive acids, such as cyclopropenoid acids, which result in high epoxide values by the usual methods, do not interfere. Results obtained by this spectrophotometric method are compared, for some oils, with those obtained by the usual chemical methods of epoxide determination.

The increasing evidence of a widespread occurrence of epoxy acids in fats makes desirable the development of more sensitive and more specific methods for their determination.

Currently, the most widely used methods for the estimation of epoxy compounds depend on the uptake of hydrogen halide by the epoxy group. The direct titration of epoxy groups with hydrogen bromide in acetic acid now constitutes a standard procedure (1, 2). This method, however, gives fallacious results with oils containing α,β-unsaturated ketones (3), cyclopropenes (4), or conjugated dieneols (4). The determination, by back titration, of the uptake of hydrogen chloride from anhydrous ether solution (5) or from dioxan solution (3) is less likely to give spurious results, but does so with α,β-unsaturated ketones, such as are found in autoxidation mixtures (3). Both methods seem liable to similar interference by other reactive compounds. Newer methods in which the end point is determined argentometrically (6, 7), or potentiometrically (8), may have the same general disadvantages, although interference by readily hydrolyzable materials and by weak bases is obviated.

However, the basic fault of all these methods is that they measure the uptake of reagent by all components which will react rather than a specific product formed from epoxy groups. The sensitivity of titration methods, too, is such that values for epoxy components present in amounts of less than about 5% are not reliable unless fairly large samples are available.

Other methods for the estimation of epoxy fatty acids which have been tried include polarography (9), which was unsuccessful, and proton magnetic resonance (10), which is a useful tool only when the epoxy component exceeds about 5% of the sample. Colorimetry (11) has found application in the measurement of short-chain epoxides but has not yet been developed for use with long-chain derivatives. Glycol determina-

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tion by periodate oxidation (12), after splitting the epoxy group, offers no advantages over the conventional methods.

The preceding paper (13) describes physical methods, of high sensitivity, for the detection of epoxy acids and esters by paper-, thin-layer-, and gas-liquid chromatography, but quantitative studies by these methods require specialized techniques and may be rather tedious.

Epoxy compounds have infrared absorption in the region of 11 to 13 μ (14) which might be used for their estimation in samples containing high proportions of epoxides, such as epoxy resins (15). The absorption, however, is relatively weak, and in most vegetable oil samples, background absorption in this region is high enough to mask any band due to epoxides present in minor amounts. A near infrared band given by terminal epoxides has been used for the determination of these compounds (16).

The strong hydroxyl absorption in the 2.8 μ region, however, permits the determination of epoxides as hydroxyl derivatives after splitting the epoxy group with an acid. This method has already been used qualitatively as a proof of the presence of a small amount of epoxy acid in *Camelina sativa* seed oil (17).

Two methods described here are based on this principle and measure the products formed from chemical reactions with epoxy groups. They are specific for epoxy groups and can readily detect and measure epoxy components constituting less than 0.5% of a fat. These methods, incidentally, detect and can estimate dimorphecolic acid (9-hydroxy-*trans*-*trans*-octadec-10,12-dienoic acid) or similar compounds in seed oils, since treatment with acid causes dehydroxylation and the reduction in unassociated OH absorption can be measured.

MATERIALS

Vernonia anthelmintica (purple fleabane) seed oil was hydrolyzed with potassium hydroxide at room temperature and the mixed acids obtained after *careful* acidification. Cleavage of the epoxide group is prevented by acidification at 0° with the theoretical amount of 2 N hydrochloric acid. The methyl esters of these acids were prepared with diazomethane in ether and methyl *cis*-12:13-epoxyoctadec-9-enoate[1] isolated by fractional distillation through a spinning-band column. The product, having 5.14% oxirane oxygen by Swern's method (5) (theory 5.16%), migrated as a single spot on siliconized paper and on thin layers of silicic acid (13). It also gave a single symmetrical peak by gas-liquid chromatographic analysis (13).

cis-9:10-Epoxystearic acid (m.p. 57.5°-58°; reported 59.5°), *trans*-9:10-epoxystearic acid (m.p. 52-54°; reported 55.5°) and *cis,cis*-9:10,12:13-diepoxystearic acid (m.p. 78°-78.5°; reported 79°) were prepared by the methods of Swern *et al.* (18, 19), and their methyl esters were obtained with ethereal diazomethane.

threo-12,13-Dihydroxy oleic acid (m.p. 50°-52°; reported 53°-54°) was derived from methyl epoxyoleate by reaction with acetic acid followed by hydrolysis (20), and its methyl ester prepared with diazomethane. A mixture of methyl *threo*-12,13-chlorohydroxyoleate and methyl *threo*-13,12-chlorohydroxyoleate was prepared from epoxyoleate by reaction with anhydrous ethereal hydrogen chloride (5).

All standard substances were checked for purity by paper- and thin-layer chromatography and, where necessary, purified by adsorption chromatography on silica gel columns.

PROCEDURE

The near infrared spectra of oil samples, as 3.00%, 1.00%, or 0.30% solutions in carbon tetrachloride, were obtained on a Beckman DK-2 Recording Spectrophotometer with fused silica cells of 1 cm path length. The control settings used throughout were scanning time, 5; time constant, 0.1; and sensitivity, 2.0. At this sensitivity the nominal slit width at 2.795 μ was 0.050 mm.

Spectra were recorded of solutions of the same concentration before and after treatment of a sample with anhydrous ethereal hydrogen chloride. This treatment converts any epoxide groups to chlorohydrins and was carried out as follows: A sample of the oil (about 200 mg) was dissolved in anhydrous ether (5 ml) in a glass-stoppered bottle; 0.2 N hydrogen chloride in anhydrous ether solution (20 ml) was added, the bottle stoppered, and allowed to stand for 3 hours. The solution was then diluted with ether, transferred to a separatory funnel, and excess hydrogen chloride removed by washing four times with water. After drying with sodium sulfate, the product was recovered by removal of the solvent under reduced pressure. The untreated oil sample was also carefully dried (Na_2SO_4) in petroleum ether or ether solution, and the sample recovered before its spectrum was recorded.

The absorbance (A) at 2.795 μ was measured from a base line equivalent to the absorption between 1.50 and 1.60 μ, or from a tangent between the minima at

[1] The epoxy ring structure is indicated by a colon, e.g., 12:13-epoxyoctadec-9-enoate, which is synonymous with 12,13-epoxyoctadec-9-enoate.

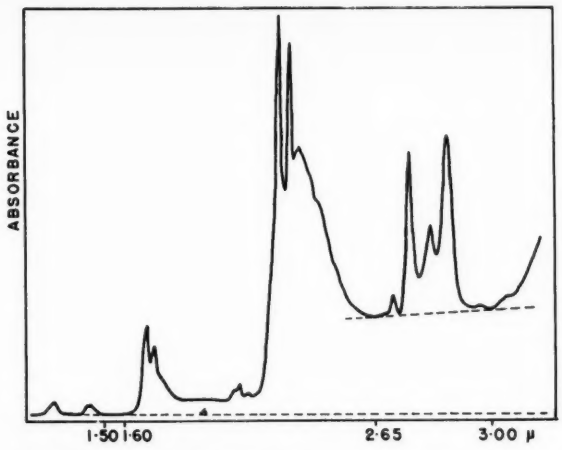

FIG. 1. Portion of near infrared spectrum of *Balanites* seed oil showing the alternative methods of obtaining a base line.

2.65 and 3.00 μ, as shown in Figure 1. The former mode of measurement is preferred since its base line, unlike the tangential base line, is unaffected by large changes of absorption in the 2.8 μ region. If solutions of the same concentration are used, the increase in absorbance (A_t-A_u) at 2.795 μ for each pair is obtained by difference and the absorptivity[2] due to chlorohydrin calculated. If different concentrations are used, the absorptivities of the original and treated samples at 2.795 μ must each be calculated and the difference obtained. The concentration of epoxy components is then calculated, as percentage of epoxyoleic acid, or as percentage of oxirane oxygen, from a calibration curve of known mixtures or by relation to the absorptivity, similarly measured, of the pure chlorohydrins:

$$\text{Epoxy content (as per cent epoxyoleic acid)} = \frac{a_t - a_u}{0.175}$$

where a_t = absorptivity at 2.795 μ of treated oil
a_u = absorptivity at 2.795 μ of untreated oil
and 0.175 = absorptivity of pure chlorohydroxy oleic acid, calculated from absorptivity of the methyl ester.

[2] The terms "absorption," "absorbance," and "absorptivity" are used according to the Joint Committee on Nomenclature in Applied Spectroscopy, *Anal. Chem.* **24**: 1349, 1952. That is, "absorption" describes the phenomenon, "absorbance" is a measure of the phenomenon without units, and "absorptivity" is a specific measurement referring to the ratio of the absorbance to the product of concentration and optical path length.

The experimental error, including errors in weighing and in measurement, is estimated to be 0.2% epoxyoleic acid for a single determination on samples containing relatively small amounts of epoxy components. The sensitivity is considered to be less than 0.2% if multiple determinations are carried out. When high proportions of epoxy acids are present in an oil, the values obtained by the procedure described above are likely to be low since introduction of a large number of chlorine groups during HCl treatment increases the average molecular weight. Thus the general background absorptivity of the untreated oil sample is higher than that of the treated sample. This can be corrected in one of two ways:

(*a*) If the uncorrected epoxy content by the above procedure is c%, as epoxyoleic acid, the absorptivity of untreated sample a_u and the molecular weights of epoxyoleic acid and chlorohydroxy oleic acid are 296.5 and 334, respectively, the following relationship holds:

$$\text{Corrected } a_u = \frac{100\, a_u}{100 - c + \frac{234}{296.5}c} = \frac{100\, a_u}{100 + 0.113c}$$

The difference between the absorptivity of the HCl treated oil at 2.795 μ and this corrected background is used to obtain the true epoxide content of the oil.

(*b*) Alternatively, the high background absorption in the untreated oil may be reduced by the ratio of the CH_2 band heights at 2.31 μ of treated to untreated oil and the difference in absorbance in the two samples calculated from this value. This method equates the number of CH_2 groups and hence the number of molecules so that a direct comparison is possible.

Assuming very accurate quantification in making up solutions of the HCl-reacted samples, the second correction method is preferable, and great accuracy in making up solutions of the unreacted oils is then unnecessary. Only in samples containing high proportions of epoxy components or having high background absorbance near 2.8 μ is such correction necessary since, in general, the difference is small. *Vernonia* oil gave an uncorrected value of 59.4%, which was raised to 59.9% by the first correction method, and to 60.2% by the second. The corresponding results for *Cephalocroton* oil were 54.2%, 54.7%, and 54.8%.

Some experiments were also carried out where epoxide groups were converted not to chlorohydrins but to 1,2-diols. This was effected by boiling the sample under reflux with glacial acetic acid for 2 hours. The acetylated oil was recovered, hydrolyzed, and the mixed acids esterified. Increase in absorption at 2.792 μ is due to the associated hydroxyl frequency of *threo*-1,2-diols produced from epoxides by this treatment.

RESULTS

Mixtures of known concentrations of pure methyl *cis*-12:13-epoxyoleate in olive oil (epoxy content 0.0%) were analyzed by these methods and calibration curves of A_t-A_u against epoxide content were drawn. Both gave straight lines through the origin (Fig. 2). From the slopes of these lines, the molar absorptivities of the pure diol and of the pure mixed chlorohydrins were calculated to be 71.4 and 58.5, respectively. These values are almost identical with those obtained from curves of the pure components (Fig. 3).

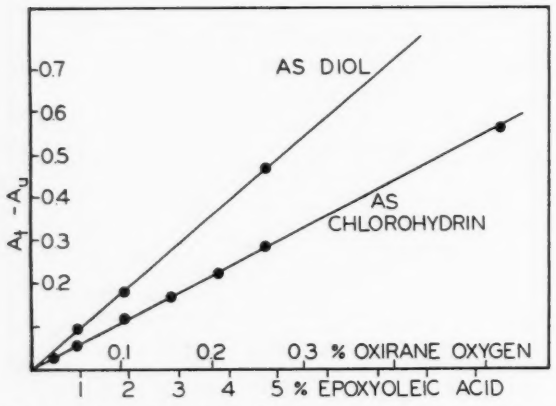

FIG. 2. Relationship of absorbance of treated sample corrected for background absorbance of untreated sample (A_t-A_u) to epoxy content of standard samples after treatment with HCl or acetic acid. Absorbance of diols measured as 4.00% solutions at 2.792 μ and of chlorohydrins as 3.00% solutions at 2.795 μ.

Molar absorptivity of the chlorohydrins from pure *cis*-9:10-epoxystearate (57.4) was the same as for those from epoxyoleate and the maximum occurred at the same wave length. The molar absorptivity of the chlorohydrins from *cis,cis*-9:10,12:13-diepoxystearate (116.0) was twice that of the monoepoxy derivatives, again at 2.795 μ. However, the *erythro*-chlorohydrins, produced by the same treatment from *trans*-9:10-epoxystearate, gave a rather different spectrum with an associated hydroxyl band at 2.793 μ of molar absorptivity 37.4 and an unassociated hydroxyl band at 2.758 μ of molar absorptivity 15.5.

The concentrations of the standard mixtures in carbon tetrachloride were 3.00% for determinations as chlorohydrins and 4.00% for the diols. If unknown samples are studied in the same concentration, any increase in absorbance at 2.795 or 2.792 μ, after hydrochloric or acetic acid treatment, respectively, may be interpolated directly on the appropriate calibration line to obtain the epoxide concentration either as per cent epoxyoleic acid or as per cent oxirane oxygen. This was the method used to obtain the values reported in Table 1.

Estimation of epoxides via the diols is, for several reasons, unsatisfactory. Free acids must be removed from the oil since they would otherwise be measured in the natural sample but not in the esters resulting from treatment. Their removal, however, may mean the loss of some epoxy material present as free acids. Furthermore, free acids are not the only constituents that will give hydroxyl absorption in the natural oil but will be removed during the hydrolysis. Mono- and diglycerides, which also have free hydroxyl groups, will be lost. These facts mean that for many oils a decrease in OH absorption will follow treatment to convert epoxy glycerides to dihydroxy esters, and small amounts of epoxy components may be overlooked because of this decrease. Although it is simple to separate triglycerides from mono- and diglycerides by silicic-acid chromatography, the separation of these components may also mean loss of some epoxy constituents.

With the chlorohydrin method the mono- and di-

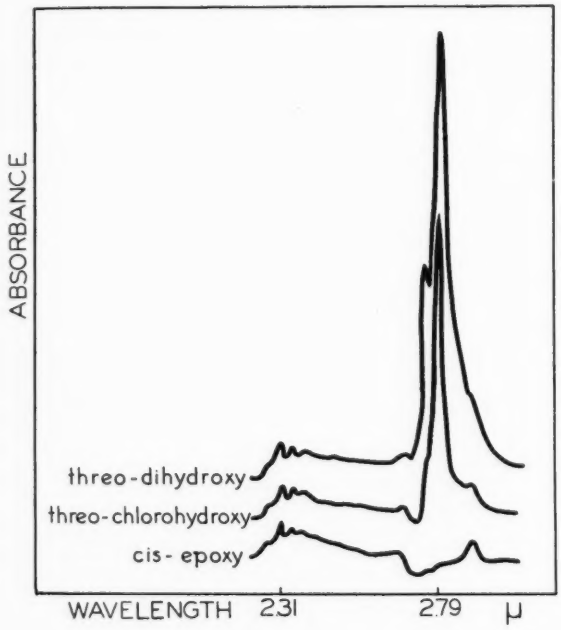

FIG. 3. Portions of near infrared spectra of methyl *cis*-12:13-epoxyoleate, methyl *threo*-12,13- and -13,12-chlorohydroxyoleate mixture and *threo*-12,13-dihydroxyoleate as 0.30% solutions in carbon tetrachloride. Molar absorptivity at 2.795 μ of chlorohydroxyoleate, calculated from this curve, is 58.5 and molar absorptivity at 2.792 μ of dihydroxyoleate is 70.1.

TABLE 1. EPOXY CONTENT OF SEED OILS BY NEAR INFRARED SPECTROPHOTOMETRY OF THEIR CHLOROHYDRINS

Plant Family	Species (Common Name)	As Epoxyoleic Acid	Oxirane Oxygen
		per cent	*per cent*
Apocynaceae	*Strophanthus sarmentosus*	0.0	0.00
Bombacaceae	*Ceiba acuminata*	0.0	0.00
Compositae	*Carthannus tinctorius* (safflower)	0.0	0.00
	Cosmos bipinnatus (cosmos)	3.2	0.17
	Helianthus annus (sunflower)	1.9	0.10
	Helianthus var. Improved Dakota	0.4	0.02
	Helianthus var. Giant Grey Stripe	0.5	0.03
	Helianthus var. Double Dwarf Sungold	0.7	0.04
	Chrysanthemum (Ann Merry mixture)	1.2	0.06
	Heliopsis scabra zinnaeflora	0.2	0.01
	Heliopsis pitcheriana	1.0	0.05
	Artemisia absinthium (wormwood)	14.9*	0.81*
	Cynara cardunculus (Argentine thistle)	1.1	0.06
	Dimorphotheca aurantiaca (Cape marigold)	0.6*	0.03*
	Vernonia anthelmintica (purple fleabane)	60.2	3.23
Cruciferae	*Brassica campestris* (rape)	0.0	0.00
Cucurbitaceae	*Apodenthria undulata*	0.7	0.04
Euphorbiaceae	*Cephalocroton cordofanus*	54.8	2.94
	Sapium sebiferum (stillingia)	0.0	0.00
Gramineae	*Zea Mays* (corn)	0.0	0.00
Leguminosae	*Calliandra eriophylla* (fairy dusters)	5.9*	0.32*
Linaceae	*Linum usitatissimum* (linseed)	0.0	0.00
Malvaceae	*Hibiscus cannabinus* (kenaph)	1.8	0.10
Oleaceae	*Olea europea sativa* (olive)	0.0	0.00
Punicaceae	*Punicum granatum* (pomegranate)	0.0	0.00
Sterculiaceae	*Brachychiton acerifolius*	0.2	0.01
Umbelliferae	*Petroselinum sativum* (parsley)	0.0	0.00
Zygophyllaceae	*Balanites aegyptica* (lalob)	3.5	0.19

* These values were estimated because the presence of conjugated diene hydroxy acid made direct measurement impossible.

glycerides, free acids, etc., remain intact. Only epoxy and a few other groups react with hydrogen chloride to any extent, and only epoxides generate new hydroxyl groups on such treatment. Moreover, the hydroxyl group of a chlorohydrin is strongly hydrogen-bonded to the chlorine and the resultant peak at 2.795 μ is generally well separated from normal hydroxyl absorption near 2.76 μ, which does not interfere.

Some oils were studied by the diol method, and the results obtained (Table 2) are in fairly good agreement with those resulting from conversion to chlorohydrins. For reasons stated above, however, the diol method was abandoned in favor of the chlorohydrin method, which was used for most of this work.

DISCUSSION

It was found that the spectra of methyl oleate, of methyl linoleate, and of nine seed oils were unchanged by the action of hydrogen chloride. Thus, increased hydroxyl absorption does not result from reaction with HCl unless epoxy compounds are present.

The difference between the spectra of *threo-* and *erythro*-chlorohydrins, produced by HCl treatment of *cis-* and *trans*-epoxides, respectively, means that the method of determination, as outlined here, is limited to mixtures containing *cis*-epoxides. Since no *trans*-epoxy acid has as yet been shown to occur naturally, the utility of the method for studies of natural oils or epoxidized natural oils is not affected by this limitation. Autoxidation mixtures, however, may contain both *cis-* and *trans*-epoxides and the differences in the spectra of the chlorohydrins from these must be borne in mind in any quantitative determination on such mixtures.

Comparison of values obtained by this method and by the methods of Durbetaki and of Swern (Table 2) shows that the last two methods give values which are higher than ours in many instances. The more reactive hydrogen bromide reagent of Durbetaki, in most cases, gives values showing the greatest difference from ours. Many oils are known to contain acids which interfere in conventional epoxide determinations (5). *Dimorphotheca* oil contains a high proportion of 9-hydroxy-*trans*-*trans*-10,12-octadecadienoic acid (21),

TABLE 2. COMPARISON OF RESULTS OBTAINED BY VARIOUS METHODS OF EPOXIDE DETERMINATION

Oil	Epoxide Content as Per Cent Epoxyoleic Acid			
	I.R. (Chlorohydrins)	I.R. (Diols)	Durbetaki*	Swern
Vernonia	60.2			63.1
Cephalocroton	54.8			57.2
Cynara	1.1	1.0	1.5	
Apodenthria	0.7			1.2
Stillingia	0.0			0.3
Hibiscus	1.8		4.8	2.9
Ceiba	0.0		1.7	2.0
Brachychiton	0.2		11.0	6.0
Cosmos	3.2	3.2	6.6	7.2
Helianthus	1.9		3.1	9.5
Artemisia	14.9†	15.0†	23.0	20.1
Dimorphotheca	0.6†	1.5†	52.0	30.7
Balanites	3.5		7.9	5.5
Calliandra	5.9†	7.6†	11.0	8.3
Heliopsis pitcheriana	1.0			2.1
Chrysanthemum	1.2			3.1

* Determinations using this method, on the same seed oils, were carried out by F. R. Earle's group at the U.S. Department of Agriculture, Northern Regional Laboratory, Peoria, Ill.

† These values were estimated.

and we have shown (22) that isomers of this acid are the cause of high epoxide values given by the oils *Artemisia, Cosmos* and *Helianthus* (family Compositae), *Calliandra* (Leguminosae), and *Balanites* (Zygophyllaceae). This particular type of reactive acid, containing a vicinally unsaturated hydroxyl structure, loses hydroxyl on treatment with hydrogen halides in organic solvents. The decreased intensity of the unassociated hydroxyl band at 2.762 μ after such treatment enables the concentration of such acids to be estimated concurrently with any epoxy components by the chlorohydrin method here described (22).

When both epoxy and reactive hydroxy acids were present together in an oil and one or the other was in high concentration ($>$ 10%), the large changes in absorption in the 2.8 μ region after HCl treatment made a direct measurement of their concentrations impossible. In these cases concentrations were estimated, making allowances for the interfering bands. The patterns of the interfering bands were constructed, with reference to the absorption curves of other oils showing similar absorption at 2.83 and 2.88 μ. The amounts of absorption due to reactive hydroxy acid in the original oil sample and caused by chlorohydrins in the acid treated sample were then estimated. Although these estimations were carried out on this rather arbitrary basis, the values so obtained for epoxy acid content and for diene hydroxy acid content were in good agreement with the values obtained by other methods (22). The ease of qualitative detection of one such acid in the presence of the other is in no way impaired, since their hydroxyl absorption bands are clearly resolved. In addition to the above six oils, where we have verified the presence of these acids by chromatographic and spectrophotometric studies (22), measurements indicate that *Heliopsis pitcheriana* and the mixed *Chrysanthemum* sample (both of the Compositae) contain a vicinally unsaturated hydroxy acid in a concentration of 0.8% and 0.6%, respectively.

Members of the families Sterculiaceae, Malvaceae, and Bombacaeae contain cyclopropene acids (23, 24, 25) which are reactive toward hydrogen halides. Acids of this type are probably responsible for all the apparent epoxide value of the *Ceiba* oil and for most of it in the *Brachychiton* and *Hibiscus* oils, as determined by the Durbetaki method.

These results demonstrate that the method for the detection and estimation of epoxy components by near infrared spectrophotometry of the chlorohydrins has much greater specificity and sensitivity than the methods in general use. Much smaller samples are required and the procedure is simple and rapid.

We are grateful for the co-operation of F. R. Earle, of the U.S. Department of Agriculture, Northern Regional Laboratory, Peoria, Illinois, in providing us with samples of many of the oils studied and in making available to us the results of epoxide determinations by the Durbetaki method on these oils. We also wish to thank S. B. Penick and Company, New York, for their gift of *Vernonia anthelmintica* seeds. The technical assistance of G. C. Miller is gratefully acknowledged.

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Volume 2
Number 1

Plasma free fatty acids in fasting vitamin C-deprived guinea pigs

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SUMMARY

Plasma concentrations of free fatty acids (FFA) were measured in normal and scorbutic guinea pigs. In animals deprived of vitamin C for 15 or 25 days, FFA concentrations were higher than normal after a 5-hour fast. Conversely, they were lower than normal after a 29-hour fast. Plasma FFA concentrations were normal after a 29-hour fast in animals deprived of vitamin C for 25 days, but then given vitamin C at the beginning of the fasting period.

In the course of recent studies a possible effect of vitamin C on plasma free fatty acids (FFA) was noted.[1] Hypophysectomized rats receiving a chow diet had lower fasting FFA concentrations than did normal rats. Addition of oranges to their diet seemed to raise the FFA toward normal. It was decided to study this phenomenon in guinea pigs which, unlike normal rats, require dietary vitamin C.

Debons and associates (1) have shown that liver slices from fasting scorbutic guinea pigs liberate relatively few ketone bodies, despite decreased utilization of ketones, and despite the potential for excessive liberation of ketones if octanoic acid is added to the system. They suggest that this may be the result of a defect in the production of fatty acids from liver lipids. Their data are also consistent with a decreased transport of fatty acids to the liver. Since plasma FFA are a major means of fatty acid transport, it seemed probable to us that fasting scorbutic guinea pigs would have relatively low plasma FFA.

METHODS

Male NIH stock, mixed color, guinea pigs weighing from 221 to 329 g were given tap water and a commercial powdered ascorbic acid-free diet (Nutritional Biochemicals, Inc.) *ad libitum*. Every morning each animal was given a 0.08 ml intraperitoneal injection. "Normal" animals received 40 mg of ascorbic acid in solution (Lederle). "Scorbutic" animals received normal saline. Groups of normal or scorbutic animals were housed in separate cages, six per cage. The container from which they ate was six inches from the floor of the cage. Food intake was not measured, since it is well established that guinea pigs deprived of vitamin C eat much less than control animals (2).

Fifteen or 25 days after starting the experiments, the animals were sacrificed between 4 P.M. and 5 P.M. Food, but not water, had been removed from the cages at 11 A.M. the same day ("5-hour fast"), or the day before ("29-hour fast"). One group of 25-day scorbutic, 29-hour fasted animals received injections of 40 mg of ascorbic acid, instead of the usual saline, immediately after food was withdrawn ("25-day scorbutic plus vitamin C").

Animals were sacrificed and blood from the severed neck collected for 30 seconds through silicone-coated funnels into silicone-coated test tubes containing 3 drops of heparin. Blood samples were immediately placed in ice. When different experimental groups were sacrificed on the same day, the order of sacrifice alternated between groups.

Plasma FFA were determined by extraction of 1 ml of plasma for 24 hours in a mixture of 2,2,4-trimethylpentane (iso-octane), glacial acetic acid, and acetic anhydride, followed by titration against 0.02 N NaOH after two washings (3). The method is similar to the modification of Gordon's method described by Shafrir (4).

[1] P. S. Mueller, unpublished observations.

RESULTS AND DISCUSSIONS

The mean cumulative changes in body weights of normal and scorbutic animals are plotted against time in Figure 1. Average group weights in grams at day

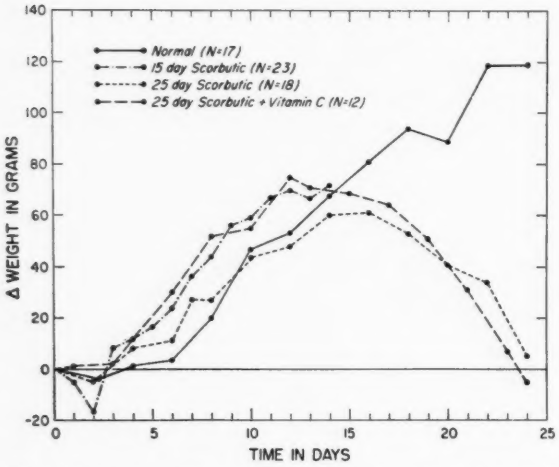

FIG. 1. Average cumulative change in body weight of control and scorbutic guinea pigs.

zero were: normal, 276; 15-day scorbutic, 240; 25-day scorbutic, 281; and 25-day scorbutic plus vitamin C, 249. Scorbutic animals gained normally for about 15 days, then lost weight. It was obvious that they began to eat less than the normal animals. They became weak and lethargic, often unable to raise themselves high enough to eat, but none died before sacrifice. No hemorrhagic phenomena were detected upon gross inspection of the skin and viscera. One normal animal died on the twenty-second day of intussusception caused by a small bowel tumor. The plasma of one scorbutic animal was lost through a laboratory error.

Plasma FFA values of various experimental groups appear in Table 1. After a 5-hour fast, normal animals had lower FFA values than did 15-day ($p < .01$) or 25-day ($p < .05$) scorbutic animals. Conversely, after a 29-hour fast, normal values were higher than in either 15-day or 25-day ($p < .05$) scorbutic animals.

Thus, prolonging the fast by 24 hours resulted in a marked increase of FFA in normal animals, but did not cause a significant increase of FFA in either group of scorbutic animals. Fifteen-day and 25-day scorbutic animals did not differ significantly from each other after comparable fasts, although values for the latter group were slightly lower.

When 25-day scorbutic animals had received an injection of vitamin C immediately after food had been withdrawn, 29-hour fasting FFA values were normal. There is no overlap between this group and the other 25-day scorbutic groups, in which comparable weight loss occurred ($p < 0.001$). After the results reported had been obtained, it was decided to check the possibility that the injection of excessive quantities of vitamin C might have altered the normal response of FFA to fasting. Groups of six animals, maintained since weaning on a chow and kale diet without injections, and weighing the same as the original vitamin C-injected normals, were sacrificed after 5- and 29-hour fasts. FFA concentrations were $0.44 \pm .07$ meq/1 and $0.93 \pm .12$meq/1, respectively; values similar to those observed in the "normal" animals of the original study.

The findings in the five groups of animals subjected to a 29-hour fast are in agreement with the initial hypothesis. Vitamin C deficiency impairs the process by which high plasma FFA concentrations occur during a prolonged fast. In the light of the finding that scorbutic guinea pigs have more body fat than do pair-fed control animals (2), and the previously cited work of Debons *et al.* (1), one may assume that there is impairment of mobilization of FFA from adipose tissue. The impairment appears to be abolished within 29 hours when vitamin C is again provided.

The relatively high plasma FFA in scorbutic 5-hour fast animals was unexpected. It might be argued that if scorbutic impairment of FFA mobilization was incomplete, the relatively high FFA is due to starvation in the 25-day group. However, this would not account for the 15-day group which was, as a group, gaining weight at the time of sacrifice. It is true that the rate of gain had slowed considerably in the four days before sacrifice, but if 5-hour FFA concentrations were

TABLE 1. PLASMA FREE FATTY ACID CONCENTRATION IN VARIOUS EXPERIMENTAL GROUPS

	5-Hour Fast			29-Hour Fast		
	N	FFA Mean ± S.D.	FFA Range	N	FFA Mean ± S.D.	FFA Range
		meq/liter	*meq/liter*		*meq/liter*	*meq/liter*
25-day Normal	11	0.41 ± 0.12	0.23 – 0.55	6	0.93 ± 0.27	0.67 – 1.30
15-day Scorbutic	11	0.60 ± 0.15	0.40 – 0.91	12	0.71 ± 0.15	0.52 – 1.00
25-day Scorbutic	12	0.55 ± 0.14	0.38 – 0.84	6	0.63 ± 0.08	0.52 – 0.76
25-day Scorbutic plus vitamin C	—	—	—	12	1.04 ± 0.15	0.86 – 1.37

so responsive to relative caloric deficit, one would expect to find an intraindividual correlation in this group of 11 animals, between weight change and FFA concentration. In fact, although individual changes in weight from the thirteenth to the fourteenth days ranged from +13 g to −20 g, the coefficient of correlation with FFA was only −0.123. Finally, it is difficult to see how a single fat-mobilizing process could be very responsive to a short fast, yet show very little further response to a much longer fast.

Previous investigations have described impaired glucose utilization (decreased glucose tolerance) after as few as 10 days of vitamin C deprivation (5, 6, 7). Furthermore, high plasma FFA concentrations have been observed in another condition in which glucose utilization is impaired, namely, diabetes mellitus (8). It is possible that the relatively high FFA concentrations within 5 hours of withdrawing food resulted from impaired glucose utilization. Such an explanation requires that the mechanism of FFA mobilization which is related to prolonged fasting be different from that related to decreased glucose utilization, because only the former mechanism appears to be impaired in the scorbutic state. This postulate must be tested by additional experiments.

The authors are grateful to David S. Plaut for his enthusiastic assistance in the biochemical determinations and animal care.

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Dependence of the lipolytic action of epinephrine *in vitro* upon thyroid hormone*†

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SUMMARY

The influence of thyroid hormone on the epinephrine-induced release of free fatty acids (FFA) from rat epididymal adipose tissue was studied *in vitro*. Untreated tissue from euthyroid animals released only small amounts of FFA. However, the tissues responded to treatment with epinephrine with a significant increase in the rate of release of FFA, confirming observations previously reported by others. In the case of fat pads removed from hypothyroid animals, no increase in the rate of release of FFA was observed after epinephrine; in the case of fat pads removed from hyperthyroid animals, the epinephrine-induced release of FFA was markedly exaggerated. This thyroidal enhancement of epinephrine action on the fat pad was not evident when tissues were removed from euthyroid animals 3 hours after intraperitoneal injection of triiodothyronine, but it was maximal in tissues removed after 15 hours from animals that had received repeated intraperitoneal injections of triiodothyronine. When triiodothyronine was added *in vitro* to fat pads from euthyroid rats, the basal release of FFA was not affected nor was the normal response to epinephrine altered. These studies show that the thyroid hormone is essential for the epinephrine-induced release of FFA from adipose tissue.

Accelerated mobilization of fat in hyperthyroid subjects is associated with an increase in the concentration of nonesterified, or free fatty acids (FFA), in plasma (1). The administration of epinephrine is also associated with an increase in the plasma FFA level *in vivo* (2, 3, 4) and, in addition, with lipolysis (5) and the release of FFA from adipose tissue *in vitro* (6, 7). Because the calorigenic effect (8, 9) and many circulatory effects (10) of epinephrine are modified by the thyroid status of the animal, we have investigated the relationship between the action of epinephrine and thyroid hormone on the release of FFA from adipose tissue.

In the present study it has been found that the response of isolated adipose tissue to epinephrine requires the mediation *in vivo* of thyroid hormone.

MATERIAL AND METHODS

Male Sprague-Dawley rats weighing approximately 275 to 325 g were used for all experiments. They were maintained on Purina Rat Chow and drinking water *ad libitum*. Rats were arranged in three groups: (*a*) hypothyroid, treated with 0.05% propylthiouracil in their drinking water; (*b*) euthyroid; (*c*) hyperthyroid, treated with 0.2 μg L-triiodothyronine per 100 g of body weight subcutaneously daily. The L-triiodothyronine was dissolved in 0.1 N NaOH and diluted with isotonic saline to give the desired dose in 0.1 ml solution (11); the final concentration of alkali was approximately 0.001 N. Groups (*a*) and (*b*) received control injections of the solvent with L-triiodothyronine omitted. After 11 to 15 days, without preliminary fasting, animals from each group were sacrificed by means of a sharp blow on the head and section of the spinal cord. The epididymal fat pads were excised and placed in ice-cold isotonic saline prior to treatment as described below under "*In Vitro* Conditions." The thyroid glands were dissected free of fascia, removed, and weighed on a Roller Smith Torsion Balance. Blood samples were also taken at this time, for measurement of the concentration of total cholesterol (12).

Another group of animals was given L-triiodothyronine by intraperitoneal injection at 3-hour intervals. These animals were prepared, and epididymal fat pads removed for *in vitro* study in the manner described

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† Presented at the fall meeting of the American Physiological Society, Urbana, Illinois, September, 1959 (*Physiologist* 2: 31, 1959).

above, at 3 hours or at 15 hours after the first intraperitoneal injection.

In Vitro Conditions. Portions of epididymal fat pads weighing approximately 200 mg were cut free hand, and incubated in 4 ml of a Krebs-Ringer Bicarbonate Buffer (13) containing 5% Albumin (Bovine, Fraction V, Armour Co.). This buffer-albumin mixture had been previously adjusted to pH 7.40 ± 0.02. The flasks, containing tissue and medium, were exposed to a stream of 95% oxygen-5% carbon dioxide for 1 minute, stoppered, and placed in a shaking water bath at 37°. Zero time control flasks were allowed to equilibrate for 15 minutes; all other flasks were incubated for 4 hours following a similar initial equilibration period, that is, for a total period of 4.25 hours. Epinephrine[1] (2.5 μg, 0.0137 μmoles) was included per each ml of buffered medium of selected flasks at zero time; in these flasks, therefore, the initial concentration of epinephrine was 13.7 μM. Duplicate aliquots were taken from the incubation medium of control flasks at the end of the 15-minute equilibration period and 4 hours later, that is, after 4.25 hours of total incubation time. The FFA concentration was determined by the method of Dole (2).

RESULTS

The relation between the amount of FFA released from the epididymal fat pad incubated under the conditions outlined above and the dose of epinephrine added to the incubation medium is shown in Figure 1. FFA release was roughly proportional to the initial concentration of epinephrine over a range from 0 to 12.5 μM, but did not increase when greater amounts of epinephrine were added to the medium, nor when 1 to 3 μmoles of ascorbic acid was included in the medium to prevent oxidation of epinephrine. Therefore, the standard epinephrine challenge employed in this study, 2.5 μg (0.0137 μmoles) per ml of incubation medium, was capable of producing a maximal release of FFA from fat pads taken from euthyroid animals.

Figure 2 shows a comparison among the three groups of animals studied (euthyroid, hypothyroid, and hyperthyroid) with respect to the release of FFA from the epididymal fat pad, both in the basal state and in response to epinephrine challenge. In the absence of epinephrine, the release of FFA from fat pads taken from euthyroid rats averaged 3.22 (± 1.36) μmoles per g of fat pad per 4-hour incubation period. The basal release of FFA from adipose tissue taken from hyperthyroid rats averaged 10.81 (± 3.56) μmoles per g of fat pad per 4-hour incubation period, and was significantly greater ($p < 0.05$) than the basal FFA release from adipose tissue taken from the euthyroid animals. Conversely, the basal release of FFA from fat pads taken from the hypothyroid animals averaged 1.54 (± 1.62), and was less than the basal FFA release from the pads taken from the euthyroid group of rats; however, the difference was not significant ($p > 0.30$).

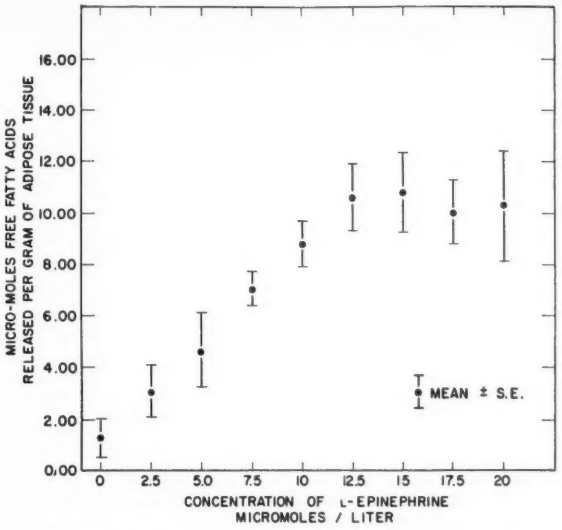

FIG. 1. Relation of the release of FFA from adipose tissue to the concentration of epinephrine in the medium.

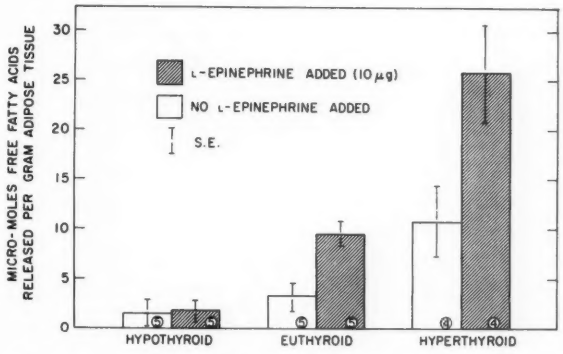

FIG. 2. Basal release and epinephrine-stimulated release of FFA from adipose tissue taken from hypothyroid, euthyroid, and hyperthyroid rats. The encircled numbers indicate the number of animals studied in each group.

[1] This preparation of epinephrine has been recently shown to have the D-configuration, although it is levorotatory. Therefore, it should be referred to as D(−)-epinephrine, instead of L-epinephrine, as given in the United States Pharmacopeia (14).

TABLE 1. EFFECT OF *in Vivo* EXPOSURE TO L-TRIIODOTHYRONINE ON THE ACTION OF L-EPINEPHRINE ON ADIPOSE TISSUE *in Vitro*

Group	Treatment	μmoles of FFA Released per G Adipose Tissue	
		No L-Epinephrine	10μg L-Epinephrine
Euthyroid	None*	2.10 ± 0.30†	6.90 ± 0.31
	L-Triiodothyronine (50μg)‡ (1 intraperitoneal injection only)	3.84 ± 0.64 (p<0.05)	7.86 ± 0.74 (p>0.05)
	None*	1.44 ± 0.12	6.97 ± 1.88
	L-Triiodothyronine (50μg)§ (every 3 hours for 12 hours)	2.43 ± 0.29 (p<0.01)	23.04 ± 3.33 (p<0.01)

* Two groups of four animals were injected intraperitoneally with saline-NaOH solvent containing no hormone.

† Mean and standard error.

‡ Two groups of four animals were sacrificed 3 hours following the injection of L-triiodothyronine, epididymal fat pads excised and treated under *in vitro* conditions.

§ Two groups of six animals were sacrificed 3 hours following the last injection of L-triiodothyronine, epididymal fat pads excised and treated under *in vitro* conditions.

Following challenge with 0.0137 μmoles of epinephrine per ml of medium, FFA release averaged 9.45 (± 1.21) μmoles per g of fat pad per 4-hour incubation period in the case of the euthyroid animals. This response was significantly less ($p < 0.01$) than that observed when fat pads from hyperthyroid rats were treated in an identical manner. FFA release from the adipose tissue taken from the latter group averaged 25.7 (± 5.0) μmoles per g of fat pad per 4-hour period. This exaggerated lipolytic effect observed when fat pads from hyperthyroid rats were challenged with epinephrine contrasted sharply with the failure of the fat pads removed from the hypothyroid animals to exhibit any response to epinephrine. The release of FFA from "hypothyroid" adipose tissue following challenge with epinephrine averaged 1.72 (± 1.2) μmoles per g of fat pad per 4-hour period, and therefore did not differ significantly from the basal rate of FFA release from fat pads observed in the experiments in which epinephrine was not included in the medium (Fig. 2).

The thyroidal enhancement of the lipolytic action of epinephrine on adipose tissue was not evident when tissues were removed from euthyroid animals 3 hours after the injection of 50 μg of L-triiodothyronine intraperitoneally, but was maximal in tissues removed from animals that had received four such injections at 3-hour intervals (Table 1).

In a separate group of experiments, L-triiodothyronine was added to epididymal fat pads *in vitro*, both in the presence and absence of epinephrine; and, conversely, epinephrine was added to epididymal fat pads *in vitro*, both in the presence and absence of L-triiodothyronine.

The addition of L-triiodothyronine *in vitro* did not alter the basal release of FFA from adipose tissue of euthyroid animals and did not affect the normal response to 10 μg (0.055 μmoles) of epinephrine (Table 2). Nor did the addition of epinephrine evoke any greater release of FFA from fat pads exposed to L-triiodothyronine *in vitro* than from fat pads that had not been exposed to L-triiodothyronine.

Additional evidence for the status of the euthyroid, hypothyroid, and hyperthyroid animals (Fig. 1) was obtained by determining the thyroid weights, calculated as mg of thyroid tissue per 100 g of body weight, which proved to be as follows: euthyroid 5.25 ± 0.22, hypothyroid 17.13 ± 1.61, hyperthyroid 3.47 ± 0.28. The concentration of total cholesterol (expressed as mg/100 ml) in the serum of the three groups averaged as follows: euthyroid 81.8 ± 8.6, hypothyroid 89.5 ± 17.6, hyperthyroid 67.0 ± 7.3.

DISCUSSION

The foregoing results show that thyroid hormone is

TABLE 2. ADDITION OF L-EPINEPHRINE AND L-TRIIODOTHYRONINE *in Vitro**

L-Epinephrine (μg)	0	0	0	10	10	10
L-Triiodothyronine (μg)	0	1	5	0	1	5
μmoles of FFA released per g adipose tissue	2.08 ± 0.48†	1.57 ± 0.35	1.83 ± 0.55	6.48 ± 0.82	7.75 ± 1.26	7.71 ± 0.89
p		>0.05	>0.05		>0.05	>0.05

* Values represent the results of 24 experiments conducted with adipose tissue taken from four euthyroid animals.

† Mean ± standard error.

required for the epinephrine-induced release of FFA from adipose tissue. This phenomenon depends on the exposure of the adipose tissue to a critical minimum level of active thyroid principle *in vivo* for a period of several (more than 3 but less than 15) hours prior to challenge with epinephrine *in vitro*. In other words, the enhancement of the lipolytic action of epinephrine on adipose tissue depends on adequate treatment of the whole animal with triiodothyronine prior to excision of the adipose tissue for *in vitro* study. In addition to the requirement of minimal amounts of thyroid hormone for even a minimal effect of epinephrine on the release of FFA from adipose tissue, it is clear that when the hormone is present in excessive amounts, the effect of epinephrine is strikingly exaggerated.

These findings are in accord with the report that the epinephrine-induced *in vivo* mobilization of FFA in rhesus monkeys is dependent upon optimal thyroid function (15). It is noteworthy that the increased mobilization of FFA from fat depots in response to fasting and growth hormone does not show a similar thyroid requirement (15, 16). Shafrir *et al.* (17) also failed to observe an elevation in plasma FFA after the administration of epinephrine to hypophysectomized dogs, confirming the findings of Goodman and Knobil (15). However, Shafrir and Steinberg (18) have reported subsequently that adrenalectomy alone abolished the epinephrine-induced increase in the plasma level of FFA in dogs *in vivo*, and treatment with cortisone restored this response, indicating a role for cortisone in the response of adipose tissue to epinephrine. They also noted that cortisone treatment alone restored the response of the plasma FFA fraction to epinephrine in hypophysectomized dogs. These authors pointed out, however, that the time interval between hypophysectomy and the execution of their studies was too short to exclude the persistence of thyroid function in their animals.

The mechanism by which the thyroid hormone modifies the capacity of epinephrine-stimulated adipose tissue to release FFA is not clear. It is possible that thyroidal inhibition of adipose tissue enzymes involved in degradation of epinephrine may be a significant factor in view of the fact that there is evidence of thyroidal inhibition of monoamine oxidase activity (19, 20, 21). Also, the fact that thyroidectomy increases the hypoglycemic response to insulin (22) suggests that a thyroid-insulin antagonism may be involved. However, we consider it more likely that this thyroid effect is due largely or entirely to an increase in the content of an epinephrine-activated lipase of adipose tissue, or to an increase in the availability of a cofactor required for activation of this enzyme, or to an increase of both (23).

We are grateful to Dr. A. E. Heming, of Smith Kline & French Laboratories, Philadelphia, Pennsylvania, for generous amounts of L-triiodothyronine (Cytomel®) used in these experiments.

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On the turnover of long-chain fatty acids in plasma

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SUMMARY

The turnover of fatty acids released by lipolysis in the body was estimated by measuring the lipolytic activity of plasma and the rise in fatty acid concentration after an injection of heparin. The mixture of fatty acids liberated endogenously appeared to be cleared at the same rate as has been previously reported for albumin-bound, C^{14}-labeled fatty acids.

Long-chain fatty acids[1] in plasma appear to transport a large quantity of material and energy (1). Their flux, as estimated from the turnover of tracer molecules (Table 1) and from the concentration of fatty acids in plasma (about 800 μeq per liter), amounts to about 250 μeq per liter per minute, or in caloric terms to about 0.6 calorie per liter per minute. Whether the flux actually delivers all of this energy directly to working cells, or whether, as appears more likely, it is in part a mixing process involving lipids of plasma and tissue, remains uncertain. In either case the apparent turnover is impressively rapid.

These estimates, however, are open to the objection that they involve only the clearance of fatty acids carried in artificially prepared fatty acid-protein complexes (2). It has seemed reasonable to believe that tracer acids injected in this form will blend indistinguishably with the native fatty acids carried on blood proteins, but the possibility remains that a complex prepared in a test tube might not be quite native, and might be cleared at an abnormal rate. An additional objection to the tracer technique is that it measures the clearance of only a single acid. Although the acids that have been measured are the major components of the fatty acid mixture (3), it is admittedly an approximation to combine the turnover of a subfraction with the concentration of a whole mixture for an estimate of total flux.

The present study has provided an estimate of the turnover of the fatty acid mixture released by lipolysis in the body. As is well known, an injection of heparin in lipemic subjects causes a transient rise in fatty acid concentration (4). If it is assumed that the rise is wholly due to lipolysis and that the clearance of fatty acids is independent of concentration at levels above 700 μeq per liter (5), their turnover can be calculated from the rate of lipolysis and the changes in concentration:

$$K = \frac{P - C'}{\Delta C} \times 10^2$$

In this expression K (per cent per minute) is the turnover rate; P (μeq per liter per minute) is the rate of lipolysis as determined by incubating a sample of postheparin blood at 37°; C' (μeq per liter per minute) is the rate of change of fatty acid concentration as determined in serial samples of blood taken with an inhibitor (6), diethyl-*p*-nitrophenyl-phosphate,[2] to prevent *in vitro* lipolysis (7); and ΔC (μeq per liter) is the increment in concentration above the preheparin base line. Each of the values (P, C', ΔC) refers to a given instant of time; blood taken for determination of P has a stable lipolytic activity as shown by a linear

[1] The term "long-chain fatty acids," or (when the chain length is understood) simply "fatty acids," is used in the present paper as a synonym for "nonesterified" or "unesterified" fatty acids. The term "free" fatty acids has been avoided since the fatty acids in question are not free until separated from protein by laboratory methods. The short-chain fatty acids in plasma might be considered as free, but usually they are excluded from the category of "free fatty acids."

The simple term "fatty acids" should be unobjectionable since molecules linked to glycerol or cholesterol by a covalent bond have lost their acid function. Esterified compounds are fatty esters, not acids; if a monocarboxylic structure is acidic, then obviously it is not esterified.

[2] Also known as Compound E 600, or Paraoxan. Obtained from Dr. Schrader, Farbenfabriken Bayer, Wuppertal-Elberfeld, West Germany.

TABLE 1. TURNOVER OF FATTY ACIDS AS ESTIMATED FROM THE CLEARANCE OF C^{14}-LABELED, ALBUMIN-BOUND FATTY ACID

Species	Acid	Half Time	Turnover	Ref.
		min	*%/min*	
Dog	Palmitic	2.0	35	(9)
Dog	Palmitic	2.1	33	(10)
Human	Palmitic	1.7	41	(11)
Human	Oleic	3.1	23	(11)
Human	Palmitic	2.5	28	(5)
Human	Oleic	2 3	30	(5)
Human	Linoleic	2.4	29	(5)

increase in concentration of fatty acids during a 30-minute period of incubation (7). This rate of production of fatty acids was assumed to be equal to that occurring in the circulating plasma at the moment of taking the blood sample. Serial blood samples taken with inhibitor for determination of fatty acid concentration of circulating plasma were plotted against time, and the values of C' (slope) and ΔC (increment of height), corresponding to the times of sampling for lipolytic activity, were estimated graphically.

Twelve medical students served as subjects. About 8:00 A.M. they ingested 240 ml of heavy cream. For the next 5 hours they took only water *ad libitum*. Starting about 1:00 P.M., samples of blood were taken at 30, 25, and 15 minutes and immediately before an injection of heparin (10 mg intravenously). After the injection, samples were taken at 5, 15, and 30 minutes. In six subjects, studied under identical conditions, but on other days, samples of blood were taken without inhibitor at 3 and 30 minutes after an injection of heparin for determination of lipolytic activity. The fatty acid concentrations in all samples were determined by a single extraction method previously described (8).

TABLE 2. TURNOVER OF FATTY ACIDS ESTIMATED FROM LIPOLYTIC ACTIVITY AND THE RISE OF FATTY ACID CONCENTRATION AFTER INJECTION OF HEPARIN

Time	P	C′	ΔC	K	
min	*μeq/liter min*	*μeq/liter min*	*μeq/liter*	*per cent/min*	
3	72.8 ± 18.3	28.2 ± 6.0	141 ± 30.1	31.7 ± 15.3	28.4 ± 9.5
30	53.3 ± 12.1	−3.9 ± 3.2	228 ± 88.6	25.1 ± 11.2	

The results, and the standard errors of the mean values, are shown in Table 2. Two estimates of turnover rate were obtained: the first was calculated from the rapid rise of fatty acid concentration during the initial 5 minutes after injection of heparin, while the second was obtained from the data of the last 15 minutes, during which time the concentration had passed its peak and was falling slowly. The standard error of turnover was calculated from the variances of the three mean values (P, C', ΔC) according to the formula:

$$\sigma_K^2 = \left(\frac{\partial K}{\partial P}\right)^2 \sigma_P^2 + \left(\frac{\partial K}{\partial C'}\right)^2 \sigma_{C'}^2 + \left(\frac{\partial K}{\partial \Delta C}\right)^2 \sigma_{\Delta C}^2$$

The two values of turnover given in Table 2 are not significantly different. Combining them yields the final estimate, 28.4 ± 9.5% per minute. This value agrees remarkably well with the turnovers that have been calculated from the clearance of labeled palmitic, oleic, and linoleic acids (Table 1).

The present results thus tend to validate some of the assumptions involved in the use of tracer acids. It must be emphasized, however, that the equality of turnover rates means only that the fatty acid-protein complexes manufactured in the test tube are essentially the same as those formed in the blood stream. It remains for future work to determine whether or not this kind of complex accounts for all of the long-chain fatty acid in circulation.

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Notes on Methodology

Quantitative gas-liquid chromatography of fatty acid methyl esters with the thermal conductivity detector*

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► Thermal conductivity detectors (katharometers) are widely used in gas-liquid chromatography (GLC). In the analysis of fatty acid methyl esters, composition is often estimated from the peak area response when a katharometer is used (1, 2, 3). However, peak area is not a simple function of concentration expressed in mole per cent or weight per cent (4). Messner *et al.* (5) recently demonstrated that the relative peak area, area per mole of component divided by area per mole of standard, is a function of molecular weight and structure. In the present investigation, the relative katharometer response for a number of fatty acid methyl esters in two homologous series has been calculated by the method of least squares from experimental data. These correction factors were used in the analysis of known mixtures and in the recalculation of published experimental data on known mixtures in order to demonstrate their value in quantitative GLC analysis. Calculated correction factors were compared with a theoretical relationship, employing molecular weights.

Methyl butyrate, octanoate, and decanoate were purchased.[1] Other methyl esters were prepared in this laboratory by fractional distillation and low temperature crystallization. Methyl linolenate was further purified by chromatography on silicic acid. The purity of *cis*- and *trans*-unsaturated esters was demonstrated by infrared spectra. All samples were over 99% pure on the basis of GLC examination.

An Aerograph A-90-C gas chromatograph[2] with a Wheelco 1 millivolt recorder[3] and a 5-foot column containing G.E. SF-96 silicone resin and Chromosorb in a 1:3 ratio[2] was used. A silicone resin was selected to avoid the possibility of losses by reactions such as esterification. The temperature of the column and katharometer varied from 208° to 228°, and filament current from 200 to 250 milliamps. for different runs. Helium was the carrier gas. Peak areas were measured with a Wheelco Type A electronic integrator[3] with a maximum counting rate of 2500 cpm. The chart was run at 180 inches/hour so that the counts per peak ranged from about 60 for methyl butyrate to about 2500 for methyl behenate. These conditions were chosen in order to minimize errors in measurement of peak areas (6).

Known mixtures of the methyl esters were prepared and chromatographed. The area of each peak was compared with the area of a standard peak. Methyl palmitate, the internal standard, was assigned a calibration factor of 100. Esters of C_{14}- and C_{18}-fatty acids were compared with a methyl decanoate standard to ensure against any overlapping of peaks. Calibration data are summarized in Tables 1 and 2. The method of least squares (7) gave the following equation for relative molar response in the saturated methyl ester series:

$$R = 24.68 + 5.79\,N - 0.075\,N^2$$

where R is the relative molar response compared with methyl palmitate and N is the number of carbon atoms in the fatty acid. Values for relative response obtained from this equation were multiplied by 1.019, a factor that corrected the calculated response of methyl palmitate to 100. The effect of *cis* double bonds in the C_{18} series was calculated by the method of least squares from the experimental data in Table 2:

$$R' = 92.84 - 1.865\,D - 0.175\,D^2$$

where R' is the relative mass response and D is the number of *cis* double bonds. Values obtained for relative mass response from this equation were multiplied by the factor 1.04 in order to obtain an equivalent relative response for methyl stearate in the saturated and unsaturated series. The effect on relative response by a *trans* double bond did not correspond to the effects obtained with *cis* compounds.

Calibration factors were shown to improve the accuracy of GLC analysis. When two known methyl

* Supported in part by Research Grant H-2807 from the National Institutes of Health.

[1] Eastman Kodak Co., Rochester, N. Y.

[2] Wilkens Instrument and Research, Inc., Walnut Creek, Calif.

[3] Barber-Colman Co., Rockford, Ill.

TABLE 1. RELATIVE RESPONSE OF SATURATED METHYL ESTERS IN THE KATHAROMETER DETECTOR

Ester	Relative Response					
	Experimental			Calculated†		Theoretical‡
	Mass*		Molar	Mass	Molar	Molar
Acetate (7)§	128	±7	35	133.8	36.6	42.2
Butyrate (11)	121	±14	46	125.9	47.5	52.3
Hexanoate				120.1	57.8	61.4
Octanoate (23)	114	±9	67	115.3	67.5	70.0
Decanoate (12)	108	±6	74	111.1	76.5	78.0
Laurate (17)	105	±5	83	107.2	85.0	85.6
Myristate (11)	102.6	±3.9	92	103.5	92.8	93.0
Palmitate				100.0	100.0	100.0
Stearate (10)	93.7	±5.1	103	96.6	106.6	106.8
Arachidate (16)	91.0	±6.0	110	93.2	112.6	113.4
Behenate (23)	88.3	±5.0	116	90.0	118.0	119.8

* Relative mass response ± standard deviation of observation.
† Calculated from equation: $R = 24.68 + 5.79\ N - 0.075\ N^2$.
‡ Calculated from relationship: $(M_1/M_2)^{2/3}$.
§ Figures in parentheses represent the number of experiments.

ester mixtures were examined by GLC (Table 3), the percentage composition calculated from uncorrected areas was elevated for low molecular weight esters and decreased for high molecular weight esters. These errors were corrected by the relative response factors. Although many quantitative studies are concerned only with higher molecular weight esters, for which differences in relative response are not large (Table 1), GLC analyses of known higher molecular weight ester mixtures reported in the literature (2, 3) were improved by their recalculation with the relative response factors obtained in this study (Table 4). The percentage composition obtained for the lower molecular weight esters was decreased, while the percentage composition obtained for the higher molecular weight and unsaturated esters was increased by the calibration factors. The data of Herb *et al.* (2) were obtained with the katharometer at 235° to 240°, and an unspecified filament current while Craig and Murty (3) operated their detector at 205° with filament currents between 160 and 200 milliamps. Since the GLC analyses described in these two studies (Table 4) were improved, even though the instruments and operating conditions were different from the present investigation, the calibration factors reported in this investigation appear to have a general application.

TABLE 2. RELATIVE RESPONSE OF C_{18} UNSATURATED METHYL ESTERS IN THE KATHAROMETER DETECTOR

Ester	Relative Response	
	Experimental Mass*	Calculated† Mass
Stearate (10)‡	93.7 ± 5.1	96.6
Oleate (26)	88.2 ± 6.2	94.5
Linoleate (25)	91.0 ± 6.6	92.0
Linolenate (11)	84.8 ± 5.6	89.2
Elaidate (22)	93.2 ± 6.8	

* Relative mass response ± standard deviation of observation.
† Calculated from the equation: $R' = 92.84 - 1.865\ D - 0.175\ D^2$.
‡ Figures in parentheses represent the number of experiments.

TABLE 3. EFFECT OF RELATIVE RESPONSE ON THE COMPOSITION OF KNOWN METHYL ESTER MIXTURES DETERMINED BY GAS-LIQUID CHROMATOGRAPHY

Ester	Composition (Weight Per Cent)				
	Known	Found*	Per Cent Error	Corrected†	Per Cent Error
Butyrate	4.6	5.9	28.3	4.7	2.2
Octanoate	9.9	11.5	16.2	10.0	1.0
Laurate	10.5	11.3	7.6	10.5	0
Palmitate	25.2	24.7	2.0	24.7	2.0
Arachidate	49.8	46.6	6.4	50.0	0.4
Decanoate	11.0	12.2	10.9	10.6	3.6
Myristate	22.8	24.6	7.9	23.0	0.9
Stearate	20.5	20.7	1.0	20.7	1.0
Behenate	45.7	42.5	7.0	45.7	0

* Calculated from uncorrected areas.
† Corrected for relative response.

Hinkle and Johnsen (8) calculated from empirical data that the molar response in a katharometer is proportional to the vapor density raised to the two-thirds power. Since the density of a gas is very nearly proportional to molecular weight, it follows that:

$$\frac{R_1}{R_2} = \left(\frac{M_1}{M_2}\right)^{2/3}.$$

TABLE 4. EFFECT OF RELATIVE RESPONSE ON THE COMPOSITION OF KNOWN METHYL ESTER MIXTURES DETERMINED BY GAS-LIQUID CHROMATOGRAPHY AND REPORTED IN THE LITERATURE

Ester	Composition (Weight Per Cent)				
	Known	Found*	Per Cent Error	Corrected†	Per Cent Error
Palmitate‡	24.9	27.0	8.4	25.9	3.6
Stearate	25.0	25.3	1.2	25.1	0.4
Oleate	25.0	24.8	0.8	25.1	0.4
Linoleate	25.1	22.9	8.8	23.9	4.4
Myristate‡	5.9	6.4	8.5	5.9	0.0
Palmitate	11.8	13.8	16.9	13.0	10.2
Stearate	19.1	20.9	9.4	20.4	6.8
Oleate	23.3	23.8	2.1	23.8	2.1
Linoleate	13.3	12.5	6.0	12.9	3.0
Linolenate	15.3	12.9	15.7	13.7	10.5
Erucate	11.2	9.6	14.3	10.3	8.0
Palmitate§	6.0	6.8	13.3	6.3	5.0
Stearate	5.2	5.1	1.9	4.9	5.8
Oleate	19.1	20.0	4.5	19.6	2.6
Linoleate	58.7	58.6	0.3	59.1	0.7
Linolenate	11.0	9.6	12.7	10.0	9.1
Palmitate§	5.5	6.4	16.4	5.9	7.3
Stearate	5.6	6.0	7.1	5.7	1.8
Oleate	19.7	21.0	6.6	20.3	3.0
Linoleate	10.5	10.1	3.8	10.1	3.8
Linolenate	58.7	56.5	3.8	58.0	1.2

* Calculated from uncorrected areas.
† Corrected for relative response.
‡ Analysis reported by Herb *et al.* (2).
§ Analysis reported by Craig and Murty (3).

Relative molar responses for saturated methyl esters were calculated from this relationship (Table 1) and found in close agreement with experimental values.

The same molecular weight relationship is obtained if it is assumed that the relative katharometer response in a homologous series is proportional to the collision frequency between molecules of the sample gas in the detector, and that differences in heat transfer per collision are not large for compounds in a homologous series. The collision frequency of a gas is proportional to the average area, r^2, where r is the radius of the molecule and equal to:

$$\left(\frac{3M}{4\pi d}\right)^{1/3}$$

and d is the density in the solid state (9). Since densities for long-chain fatty acid esters are nearly equal, the relative frequency of collision for equal molar quantities of two sample gases is proportional to:

$$\left(\frac{M_1}{M_2}\right)^{2/3}.$$

Thus differences in relative response for a homologous series may be caused by differences in molar volume and therefore collision frequency. The anomalous relative response found in the unsaturated C_{18} homologous series with the introduction of a *trans* double bond (Table 2) can then be explained by the negligible effect of a *trans* double bond on molar volume. Deviations for low molecular weight compounds in the homologous series (Table 1) can result from the assumptions made in approximating molar volume or significant differences in heat transfer per collision with the sensing element.

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The direct determination of liver triglycerides

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► The level of tissue triglycerides is generally calculated by subtracting the sum of phospholipids, cholesterol, and cholesterol esters from total lipids. The various procedures are usually tedious, and can give rise to considerable error since the triglycerides may constitute only a small fraction of the total lipids. Accordingly, there is need for a simple method for the direct assay of tissue triglycerides.

Recently, Van Handel and Zilversmit (1) published a method for the direct determination of plasma triglycerides. In this method, phospholipids are separated from triglycerides by adsorption on a synthetic zeolite. The triglycerides are then extracted into chloroform and estimated from the content of esterified glycerol.

This paper describes an adaptation of the method of Van Handel and Zilversmit to the direct estimation of liver triglycerides. This simple and accurate method makes it possible to analyze a large number of tissue samples in a single day.

REAGENTS

Phosphate Buffer, M/15, pH 7.0. Mix M/15 Na_2HPO_4 solution and M/15 KH_2PO_4 solution in the proportion of 61.1 to 38.9. Other reagents are the same as those described by Van Handel and Zilversmit (1), except for a higher concentration (2.0 M) of sodium arsenite. This increase in concentration of arsenite lowers the optical density of the unsaponified blank in determinations on liver homogenates.

PROCEDURE

The procedure consists of five steps: (*a*) homogenization of tissue, (*b*) the adsorption of phospholipids onto zeolite, followed by the extraction of triglycerides into chloroform, (*c*) hydroylsis of triglycerides to fatty acids and glycerol, (*d*) oxidation of glycerol with $NaIO_4$ to formic acid and formaldehyde, and (*e*) the formation of a colored complex of formaldehyde and chromotropic acid. The last four steps are carried out essentially as described by Van Handel and Zilversmit (1) for plasma.

Homogenize 2 to 10 g tissue with 9 volumes (9 ml/g tissue) of phosphate buffer for 90 seconds in a Waring blendor, using a semi-micro container. Immediately after homogenization, transfer 1 ml of the homogenate into a 25 ml glass-stoppered, graduated cylinder containing about 4 g of activated zeolite moistened with 2 ml of chloroform. Add 18 ml of chloroform and shake by hand intermittently for about 10 minutes (the tissue triglycerides are now diluted 1:200). Filter through coarse, fat-free paper. Pipette an aliquot of filtrate, containing about 0.05 mg of triglycerides (usually 0.125 to 1 ml), into each of three glass-stoppered tubes. Then pipette 1 ml of the standard corn oil solution (0.05 mg/ml) into each of three additional glass-stoppered tubes. Evaporate the chloroform from all tubes by placing in a water bath maintained at about 80°. To two out of each three standards and unknowns, add 0.5 ml of alcoholic KOH (saponified sample); to the third standard and unknown, add 0.5 ml of 95% alcohol (unsaponified sample). Maintain tubes at 60° to 70° for 20 minutes. Add 0.5 ml of 0.2 N H_2SO_4, and remove alcohol by placing tubes in a gently boiling water bath for about 15 minutes. Cool the tubes and add 0.1 ml of periodate solution. After 10 minutes, add 0.1 ml of sodium arsenite solution. Several minutes later, add 5 ml of chromotropic acid reagent. Mix and then heat for one-half hour by placing in a boiling water bath in the absence of excessive light. After cooling, determine the optical density (O.D.) at 570 mμ.

CALCULATION OF RESULTS

Let R =

$$\frac{\text{O.D. sapon. unknown} - \text{O.D. unsapon. unknown}}{\text{O.D. sapon. corn oil stand.} - \text{O.D. unsapon. corn oil stand.}}$$

and A = volume of aliquot of chloroform extract in ml. Then triglyceride contents in milligram per gram tissue =

$$\frac{200}{A} \times R \times 0.05 = 10\,\frac{R}{A}.$$

RESULTS

Recoveries. Recoveries were determined by adding known amounts of corn oil to the liver homogenate, rehomogenizing the mixture for 90 seconds, and measuring the triglyceride content of aliquots of the resulting suspension. Recoveries from livers of dogs, rats, and squirrel monkeys averaged 85% to 105%.

Reproducibility. Replicate estimations were made of the liver triglyceride content of normal rats and of animals pretreated with alcohol, ethionine, and carbon tetrachloride (2). Although the triglyceride values ranged from 5 to 100 mg/g, the coefficients of variation were between 3.5 and 6.5.

Specificity. Estimation of the triglyceride content of four rat liver homogenates yielded values which agreed satisfactorily with those obtained on the same homogenates by silicic-acid chromatography after ethanol-ether extraction (3) (Table 1). The possibility was investigated that mono- and diglycerides, if present in appreciable amounts, might be included also in the direct assay of triglycerides. The α-monoglycerides would increase the optical density of the unsaponified blank, since the periodate oxidation yields an almost stoichiometric amount of formaldehyde. However, the typical α-monoglycerides, 1-monopalmitin and 1-monostearin, were found to be largely (more than 80%) retained by the zeolite. Since the extraction of the monoglycerides from the zeolite is largely incomplete, it may be concluded that the triglyceride content of tissues would not be significantly influenced by the monoglyceride content unless abnormally large amounts were present.

TABLE 1. TRIGLYCERIDE CONTENT OF NORMAL RAT LIVER*

Rat	Liver Triglyceride	
	Proposed Method	Silicic-Acid Chromatographic Method
	mg/g	*mg/g*
1	7.2	7.3
2	10.6	10.2
3	4.0	3.6
4	6.5	6.3

* A comparison of values of triglyceride content of normal rat livers obtained by the method proposed in this paper and by silicic-acid chromatography after ethanol-ether extraction (3). Each rat liver was homogenized with phosphate buffer, 1 ml removed for the colorimetric determination, and the triglycerides in the remaining homogenate were determined gravimetrically after extraction with ethanol-ether and isolation by silicic-acid chromatography.

In contrast, 1-3-dipalmitin, a typical diglyceride, was not adsorbed onto zeolite, but was extracted into chloroform along with the triglycerides. Values for liver triglycerides will therefore include any diglycerides present in the tissue. In normal rat liver these generally do not exceed 1 mg/g of liver.[1] We tested the possibility that a fatty liver might contain a relatively high concentration of diglycerides. A fatty liver was obtained from a rat treated with carbon tetrachloride. A portion (1.8 g) of the liver was homogenized and treated with zeolite and chloroform to remove the phospholipids. The chloroform supernatant from the zeolite was separated into the neutral lipid classes by silicic-acid chromatography (3). Only 5 mg/g of phosphate-free, nontriglyceride lipid was obtained. Analysis of this material for glycerol (steps *c* through *e* under Procedure) indicated that it consisted of glycerides. Calculated as diglyceride, the colorimetric estimation yielded a value of 4.2 mg/g, about the same as the actual weight of the analyzed lipid. In this markedly fatty liver, the diglyceride content, although elevated, was less than 4% of the triglyceride content (4.2 mg/g diglyceride; 119 mg/g triglyceride).

The diglyceride content of liver can account for the slightly higher triglyceride values obtained by the colorimetric procedure than by silicic-acid chromatography.

The 1,3-dipalmitin and 1-monopalmitin were generously supplied by Dr. D. M. Turner and had been obtained from Dr. F. Mattson. The 1-monostearin was purchased from Eastman Chemical Co.

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[1] D. Turner, personal communication.

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New Methods

► **New Methods is a listing of pertinent references to analytical lipid methods published recently. It is compiled by H. A. I. Newman and Norman S. Radin.**

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Scope: The JOURNAL OF LIPID RESEARCH, a quarterly, will publish original articles dealing with the chemistry, biochemistry, enzymology, histochemistry, and physiology of the lipids. The editors will favorably consider significant contributions in the field of lipid methodology and will encourage publication of sufficient details so that the methods can be reproduced. Clinical observations and nutritional data will be welcomed if they offer contributions in the above fields. In addition to original articles, the JOURNAL will contain review articles and a section, "Notes on Methodology," dealing primarily with minor improvements in existing methodology. Notes on Methodology will not generally require a summary or subdivision into sections. A listing of new lipid methods published in other journals will also be provided. Although articles will be published in English only, it is hoped that the JOURNAL will become a vehicle for international communication.

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Units of Mass:	
kilogram	kg
gram	g
milligram	mg
microgram	μg
millimole	mmole (not mM)
micromole	μmole (not μM)
Units of Concentration:	
molar (mole per liter)	M
millimolar	mM
micromolar	μM
Units of Length, Area, Volume, etc.:	
meter	m
centimeter	cm
millimeter	mm
millimicron	mμ
square centimeter	cm^2
liter	liter
milliliter	ml
cubic centimeter	cc or cm^3
microliter	μl
counts per minute	cpm
counts per second	cps
millicurie	mc
microcurie	μc
temperature (Centigrade only)	°

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